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ANTIMICROBIAL RESISTANCE IN THE MAJOR RESPIRATORY TRACT PATHOGENS – METHODS AND EPIDEMIOLOGY

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ACADEMIC DISSERTATION

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"The time may come when penicillin can be bought by anyone in the shops",
Alexander Fleming, Nobel lecture, 1945

ABSTRACT

The major bacterial species that cause respiratory tract infections, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* have been moderately susceptible to different antimicrobial agents in Finland, but various studies have shown that their resistance to antimicrobials is growing. Consumption of antimicrobial agents has been shown to associate positively with the development of resistance. This study examined resistance to antimicrobial agents in the above-mentioned bacterial species from the perspective of methodological issues and antimicrobial consumption.

Material and Methods: Reference isolates were used to find the most appropriate susceptibility testing method for *H. influenzae*, particularly to identify the non- β -lactamase mediated ampicillin-resistance (β -lactamase negative ampicillin resistant, BLNAR). Twenty-six Finnish clinical microbiology laboratories participated in this study.

Quality control results (2004–2006) from 21 Finnish laboratories were collected to analyze the accuracy of their susceptibility testing practices for *H. influenzae* and *S. pneumoniae*. The results were compared to the expected values provided by the standard (FiRe, Finnish Study Group for Antimicrobial Resistance) applied in Finland at that time.

Performance of an automated susceptibility testing method for *S. pneumoniae* (Vitek2® AST-GP74) was investigated using 229 isolates that had been obtained from Päijät-Häme Central Hospital (Lahti, Finland). The broth dilution method (Sensititre®) was used as the reference method. Breakpoints provided by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) were used for evaluating the results.

Macrolide resistance mechanisms among *H. influenzae* strains from three periods (1988–1991, n=204; 1999–2000, n=379 and 2006–2011, n=130) were examined. Ribosomal methylation, active efflux and mutations in the bacterial ribosome in positions critical for macrolide binding were investigated.

The effect of sulfamethoxazole-trimethoprim consumption on the resistance levels of these three respiratory pathogens was examined by comparing regional resistance (1997–2003; FINRES) by hospital districts (n=21), with the respective drug use for the preceding year expressed as the defined daily doses (DDD)/1000 inhabitants/day; Finnish Medicines Agency, Fimea). The associations were modeled using a linear mixed model with drug use as the explanatory factor and resistance (%) as the dependent variable.

Results: BLNAR *H. influenzae* strains were detected better by using the low concentration disks of ampicillin (2 µg, sensitivity 92%, specificity 90%) and of amoxicillin-clavulanic acid (3 µg, sensitivity 92%, specificity 90%) than by using the high concentration disks (10 µg and 30 µg, sensitivities 71% and 44%, specificities 88% and 96%). This was also noted in the study, which examined the quality control results. 22.8% (n=372) and 78.9% (n=218) results were found to be incorrect when the high strength-disks were used. The corresponding figures for low concentration discs were 1.5% (n=596) and 4.5% (n=484).

An automatic susceptibility testing method (Vitek2®, AST GP74) provided highly comparable results for *S. pneumoniae* with the broth dilution method. Categorical agreement (CA) was 95.2–100% depending on the drug. The total number of false susceptible results (FS) was 1.0%, minor errors (mE) 1.3%, major errors (ME) 0.1% and very major errors (VME) 1.6%. Overall essential agreement was (EA) 99.0%. The EUCAST -guidelines for susceptibility testing provides different breakpoints for penicillin and meropenem, when meningitis isolates are examined. Results of this automated method showed 2.6% very major errors in the interpretation of penicillin-results and 0.4% minor errors in meropenem-results with the meningitis breakpoints. These results must therefore be interpreted with caution, when the isolate has been obtained from cerebrospinal fluid.

Macrolide resistance among Finnish *H. influenzae* strains is very low (n=6/713, 0.8% of the total material). Among the resistant strains no mobile resistance genes (*erm*, *mef*) were detected. These genes are common in macrolide-resistant pneumococci. Mutations associated with resistance to macrolide resistance reported in other bacterial species were found. Five (5/6) resistant strains carried mutations in ribosomal proteins L4 (T64K) and L22 (E78D, DEL79GP), and in the ribosome (23S rRNA, A2058G). The only L22–mutation observed in this study has not been described previously.

Regional sulfamethoxazole-trimethoprim consumption was found to have a positive connection with resistance in *S. pneumoniae* (p=0.007) but the change of resistance was not significant (p=0.452). The change in resistance over time for *H. influenzae* was border-line significant (p=0.051), but the drug use did not explain the trend (p=0.808). The change in resistance among *M. catarrhalis* was not statistically significant (p=0.349) and there was no significant association (p=0.744) between the drug consumption and the level of resistance. The use of sulfamethoxazole-trimethoprim fell throughout the country during the investigation period.

Conclusions: The accuracy of the susceptibility testing of bacteria requires evidence-based standardization and continuous quality controlling. Clinical laboratory automation can be implemented safely in pneumococcal susceptibility testing. Increasing use of macrolide antibiotics towards the end of the 20th century has hitherto not produced significant resistance in *H. influenzae* in Finland. The impact of sulfamethoxazole-trimethoprim

consumption on resistance varies for different bacterial species. A reduction in its use in the long run has not led to a significant reduction in resistance.

TIIVISTELMÄ

Tärkeimmät hengitystieinfektioita aiheuttavat bakteerit *Streptococcus pneumoniae*, *Haemophilus influenzae* ja *Moraxella catarrhalis* ovat olleet Suomessa kohtalaisen herkkiä mikrobilääkkeille, mutta eri tutkimuksissa on myös todettu, että resistenssi on kasvusuunnassa. Mikrobilääkkeiden kulutuksella on osoitettu olevan yhteys resistenssin kehittymiseen. Tässä tutkimuksessa on tarkasteltu hengitystiepatogeenien resistenssiä menetelmänäkökulmasta sekä tutkittu lääkekulutuksen yhteyttä resistenssin kehittymiseen.

Aineisto ja menetelmät: Resistenssiltään tunnettuja *H. influenzae* kantoja käytettiin tutkimuksessa, jossa haettiin soveltuvinta menetelmää erityisesti β -laktamaasi negatiivisten, ampisilliiniresistenttien (BLNAR) kantojen löytämiseksi. Kyseessä oli suomalaisissa kliinisen mikrobiologian laboratorioissa (n=26) suoritettu monikeskustutkimus.

Suomalaisten laboratorioiden (n=21) herkkyysmääritystulosten oikeellisuutta selvitettiin keräämällä suositusten mukaisten vertailubakteerikantojen (*H. influenzae* ja *S. pneumoniae*) tulokset kolmen vuoden ajalta (2004 – 2006). Tuloksia verrattiin tuona ajankohtana Suomessa käytössä olleen herkkyysmääritysstandardin (Finnish Study Group for Antimicrobial resistance, FiRe) antamiin suositusarvoihin.

Automaattista menetelmää (Vitek2®, AST GP74) *S. pneumoniae* herkkyysmäärityksissä tutkittiin Päijät-Hämeen keskussairaalassa eristettyjen potilaskantojen (n=229) avulla. Vertailumenetelmänä käytettiin liemilaimennosmenetelmää (Sensititre®).

H. influenzae-kantojen makrolidiresistenssin mekanismeja selvitettiin kolmesta noin 10 vuoden välein suomalaisissa laboratorioissa kerätystä bakteerikantakokoelmasta (1988–1991 n = 204; 1999–2000 n = 379 ja 2006–2011 n = 130). Kannoista tutkittiin geenit, jotka johtavat ribosomien metylaatioon ja lääkkeen pumppaamiseen ulos solusta sekä lääkkeiden sitoutumiskohtaan liittyvät mutaatiot ribosomeissa.

Sulfa-trimetopriimin käytön vaikutusta kolmen hengitystiepatogeenin resistenssiin tutkittiin vertaamalla sairaanhoitopiireittäin (n=21) alueellista resistenssiä (1997–2003, FINRES) edellisen vuoden lääkekäyttöön (DDD/1000 asukasta/päivä, Finnish Medicines Agency, Fimea). Yhteyttä mallinnettiin tilastollisesti käyttämällä lineaarista sekamallia, jossa lääkekäyttö oli selittävänä tekijänä ja resistenssi (%) riippuvana muuttujana.

Tulokset: BLNAR *H. influenzae* – kannat todettiin laboratorio-olosuhteissa kiekkoherkkyysmenetelmällä selvästi paremmin käyttäen matalan pitoisuuden ampisilliini (2 µg, herkkyys 92 %, spesifisyys 90 %) ja amoksisilliini-klavulaanihappokiekkaja (3 µg, herkkyys 92 %, spesifisyys 90

%) kuin käyttämällä korkean pitoisuuden kiekkoja (10 µg ja 30 µg, herkkyys 71 % ja 44 %, spesifisyys 88 % ja 96 %). Tämä todettiin myös tutkimuksessa, jossa selvitettiin vertailukantojen tulosten osuvuutta. 22.8 % (n=372) ja 78.9 % (n=218) mittauksista johti virheelliseen herkkyysluokitukseen korkean pitoisuuden kiekkoilla. Vastaavat luvut matalan pitoisuuden kiekkoilla olivat 1.5 % (n=596) ja 4.5 % (n=484).

Automaattinen herkkyysmäärittäminen menetelmä (Vitek2®, AST-GP74) tuottaa vertailukelpoisia tuloksia *S. pneumoniae* herkkyysmäärittäyksissä verrattuna referenssimenetelmään. Tulkintatulos (S/I/R) oli yhtäpitävä 95.2 – 100 % mittauksista lääkaineesta riippuen. Vääriä S -tuloksia todettiin kokonaisuudessaan 1.1 %, vähäisiä virhetulkintoja 1.3 %, merkitseviä virhetulkintoja 0.1 % ja vakavia virhetulkintoja 1.6 %. EUCAST – herkkyysmäärittämissä standardissa selkädinnesteenäytteistä eristetyille *S. pneumoniae* -kannoille on suositeltu penisilliinille ja meropeneemille erilaisia luokittelurajoja kuin muille näytteille. Automaattimenetelmällä näitä tulkintarajoja käyttäen todettiin 2.6 % vakavaa poikkeamaa penisilliiniin ja 0.4 % vähäistä poikkeamaa meropeneemiin tuloksissa.

Suomalaisten *H. influenzae* -kantojen makrolidiresistenssi on hyvin matalaa (6/713, 0.8 % koko aineistossa). Resistenteissä kannoissa ei todettu siirtyviä resistenssigeenejä (*erm*, *mef*), jotka ovat tyypillisiä mm. makrolideille resistentillä pneumokokkikannoilla. Tässä työssä yhdellä resistentillä kannalla ei havaittu makrolidiresistenssiin liittyviä tunnettuja mutaatioita. Viidellä (5/6) resistentistä kannoista havaittiin mutaatioita ribosomaalisissa proteiineissa L4 (T64K) ja L22 (E78D, DEL79GP) sekä ribosomissa (23S rRNA, A2058G). L22-proteiinissa havaittua mutaatiota ei ole kuvattu aiemmin.

Alueellisella sulfa-trimetopriimin kulutuksella havaittiin olevan positiivinen yhteys myöhempään resistenssiin *S. pneumoniae*-lajilla (p=0.007), mutta resistenssin muutos tutkimusajanjaksolla ei ollut merkitsevää (p=0.452). *H. influenzae*-lajilla resistenssin muutos tällä ajanjaksolla oli tilastolliselta merkitsevyydeltään raja-arvoinen (p=0.051), mutta lääkekäyttö ei selittänyt resistenssin muutosta (p=0.808). *M. catarrhalis*-kannoilla ei havaittu tilastollisesti merkitsevää muutosta resistenssissä (p=0.349) eikä lääkekäytön ja resistenssin välillä todettu yhteyttä (p=0.744). Sulfa-trimetopriimin kulutus väheni koko maassa tutkimusajankohtana.

Johtopäätökset: Bakteereiden herkkyysmäärittäytulosten oikeellisuus edellyttää näyttöön perustuvaa standardointia ja herkkyysmäärittäytulostasojen seuranta tulee sisällyttää laboratorion toimintaan. Kliinisen laboratorion automaatiota voidaan edistää turvallisesti pneumokokin herkkyysmäärittäytulosten osalta. Makrolidiantibioottien kasvava käyttö 1900 – luvun lopulla ei ole toistaiseksi tuottanut merkittävää resistenssiä *H. influenzae* – kantoihin. Sulfa-trimetopriimin käytön vaikutus resistenssiin on

erilaista eri bakteerilajien kohdalla. Sen käytön väheneminen ei pitkällä aikavälillä ole johtanut merkittävään resistenssin vähenemiseen.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are hereafter referred to by the Roman numerals:

I **Kärpänoja P, Nissinen A, Huovinen P and Sarkkinen H on behalf of the Finnish Study Group for Antimicrobial Resistance (FiRe network).** 2004. Disc diffusion susceptibility testing of *Haemophilus influenzae* by NCCLS methodology using low-strength ampicillin and amoxicillin-clavulanic acid discs. *Journal of Antimicrobial Chemotherapy* 53: 660–663.

II **Kärpänoja P, Nyberg ST, Bergman M, Voipio T, Paakkari P, Huovinen P, Sarkkinen H and the Finnish Study group for Antimicrobial Resistance, FiRe-network.** 2008. Connection between Trimethoprim-Sulfamethoxazole Use and Resistance in *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. *Antimicrobial Agents and Chemotherapy* 52: 2480–2485.

III **Kärpänoja P, Nissinen A, Huovinen P, Sarkkinen H and the Finnish Study group for Antimicrobial resistance, Fire-network.** Antimicrobial susceptibility testing of *Streptococcus pneumoniae* and *Haemophilus influenzae* – Internal quality control as a quality tool on a national level. 2013. *Acta Pathologica Microbiologica et Immunologica Scandinavica* 121:561-568.

IV **Kärpänoja P, Sarkkinen H.** Comparative evaluation of automated VITEK AST GP74 and E-test gradient diffusion to broth dilution for susceptibility testing of *Streptococcus pneumoniae*. 2016. Manuscript submitted.

Some unpublished results are also presented.

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THE AUTHOR'S CONTRIBUTION

- I. Design and execution of the study, analyzing of the results and drafting of the manuscript. The co-authors revised the paper.
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ABBREVIATIONS

Amp	ampicillin
Amc	amoxicillin-clavulanic acid
ATC	anatomical therapeutic chemical classification
ATCC	American Type Culture Collection
Azm	azithromycin
BD	broth dilution
BLNAR	β -lactamase-negative, ampicillin resistant
BLNAS	β -lactamase negative, ampicillin susceptible
BLPACR	β -lactamase-positive, amoxicillin-clavulanic acid resistant
bp	base pair
BRO	β -lactamase, name from (<i>Branhamella</i>) <i>Moraxella catarrhalis</i>
BSAC	British Society for Antimicrobial Chemotherapy
CA	categorical agreement
CCCP	carbonyl <i>m</i> -chlorophenylhydrazone
CF	cystic fibrosis
CFU	colony forming unit
Cli	clindamycin
CLSI	Clinical Laboratory Standards Institute
Clr	clarithromycin
COPD	chronic obstructive pulmonary disease
CRG	Commissie Richtlijnen Gevoeligheidsbepalingen
DD	disk diffusion
DDD	defined daily dose
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
DID	defined daily doses/1000 inhabitants/day
DIN	Deutsches Institut für Normung
DNase	deoxyribonuclease
DURG	Drug Utilization Research Group (WHO)
EA	essential agreement
EARSS	European Antimicrobial Resistance Surveillance System
ECOFF	epidemiological cut-off value
EDTA	ethylenediaminetetra-acetic acid
EQA	external quality assessment
Ery	erythromycin
ESBL	extended spectrum β -lactamase
ESCMID	European Society for Clinical Microbiology and Infectious Diseases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
Fimea	Finnish Medicines Agency

FiRe	Finnish Study Group for Antimicrobial resistance
FS	false susceptible
FSR	false susceptibility rate
GTP	guanosine triphosphate
HTM	Haemophilus Test Medium
I	intermediate
IS	Iso Sensitest
IQA	internal quality assessment
MALDI-TOF MS	matrix-assisted laser desorption ionization-time of flight mass spectrometry
mE	minor error
ME	major error
MEF	middle ear fluid
MH	Muller-Hinton agar/medium
MH-F	Muller-Hinton agar for fastidious bacteria
MIC	minimal inhibitory concentration
mRNA	messenger ribonucleic acid (messenger RNA)
MRSA	methicillin resistant <i>Staphylococcus aureus</i>
NCCLS	National Committee for Clinical laboratory Standards
NGS	next generation sequencing
NT	non-typable
OM	outer membrane
Oxa	oxacillin
PBP	penicillin binding protein
PCR	polymerase chain reaction
PDD	prescribed daily doses
Pen	penicillin
PROTEKT	Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin
qc	quality control
R	resistant
ROB	β -lactamase, named after a patient
rRNA	ribosomal ribonucleic acid (ribosomal RNA)
RTI	respiratory tract infection
S	susceptible
SD	standard deviation
SFM	Comite de l'Antibiogramme de la Societe Francaise de Microbiologie
SRGA	Swedish Reference Group for Antibiotics
SxT	sulfamethoxazole-trimethoprim
Tcy	tetracycline
TEM	β -lactamase, named after the patient
THFA	tetrahydrofolic acid
Tlt	telithromycin
Tmp	trimethoprim

tRNA	transfer ribonucleic acid (transfer RNA)
TTR	time to results
UKNEQAS	United Kingdom National External Quality Assessment Services
URTI	upper respiratory tract infection
Van	vancomycin
VGS	viridans group streptococci
VISA	vancomycin intermediate <i>Staphylococcus aureus</i>
VSSA	vancomycin susceptible <i>Staphylococcus aureus</i>
WGS	whole genome sequencing
VME	very major error

1 INTRODUCTION

Increasing trend of antimicrobial resistance in bacteria that cause infectious diseases is a global problem, although resistance significantly varies between geographical regions. Today, common bacterial pathogens can be resistant to all known antimicrobial agents (Mediavilla et al., 2016; Skov and Monnet, 2016). The growing resistance has been linked to the increasing use of antimicrobials in humans (Bergman et al., 2006). Nontherapeutic use of antibiotics in food industry and antimicrobial use in veterinary medicine add the reservoir of resistant bacteria in animals. There is evidence that such resistant isolates have transferred from animals to humans (Marshall et al., 2011).

The battle against antimicrobial resistance is highly dependent on the knowledge of resistance rates for different bacterial species and also requires accurate methods to measure resistance. In Finland laboratories provide resistance data for the national FINRES report on annual basis. These annual resistance data have already accumulated and have been archived for a long period with high coverage. Finnish laboratories are also committed to using standardised susceptibility testing methods, which is expected to guarantee the quality and uniformity of the national resistance data. Methodological harmonization has proceeded lately even at the European level, therefore we can expect to get access to internationally comparable resistance data.

Respiratory tract infections (RTI) are the most common reason to prescribe antibiotics in the Finnish primary health care sector (Rautakorpi et al., 2001). Of the main respiratory pathogens, *Streptococcus pneumoniae* and its resistance to macrolides has been most studied in Finland. Latest studies of other respiratory pathogens date back to the 1990s.

This research aimed to complement the Finnish susceptibility standard, determine the quality of Finnish resistance data, examine the association between the use of sulfamethoxazole-trimethoprim and resistance in three major bacterial respiratory pathogens, and investigate the level and mechanisms of macrolide resistance in *H. influenzae*. These studies can promote the quality of Finnish resistance data among respiratory pathogens and shed some light on the difficult issue of the mechanism(s) between antimicrobial use and resistance.

2 REVIEW OF THE LITERATURE

2.1 The bacteria that cause upper respiratory tract infections

2.1.1 Major pathogens

2.1.1.1 *Streptococcus pneumoniae*

The taxonomic classification of the streptococci is presented in Table 1.

Table 1. Taxonomy of *Streptococcus pneumoniae* (De Vos et al., 2009).

Kingdom	Bacteria
Phylum	Firmicutes (low G+C content gram-positive bacteria)
Class	Bacilli
Order	Lactobacillales
Family	Streptococcaceae
Genus	<i>Streptococcus</i>
Species	<i>Streptococcus pneumoniae</i>

Bacterial species included in the genus *Streptococci* are catalase-negative, gram-positive cocci that form chains and pairs especially when grown in liquid media. The phenotypic classification of the genus *Streptococci*, still considered of value in clinical microbiology, separates the group into β -haemolytic streptococci (also called pyogenic streptococci) and non-pyogenic streptococci. The non-pyogenic (also referred to as viridans streptococci) group includes over 30 α -haemolytic, non-haemolytic and even some β -haemolytic species and is further divided into five groups on the basis of 16S rRNA gene sequencing. These five groups are: *S. mitis*, *S. anginosus*, *S. mutans*, *S. salivarius* and *S. bovis*. *S. pneumoniae* is α -haemolytic and is clustered genetically in the mitis group (Kawamura et al., 1995; Spellerberg and Brandt, 2015).

S. pneumoniae was first described in the late 1880s, in France as *Microbe septicémique du salive* and concurrently in the U.S.A. as *Micrococcus pasteurii*. When the organism was recognized to be the most common cause of pneumonia, it was termed as “pneumococcus”, but later named as *Diplococcus pneumoniae* because of its typical dyadic appearance under the microscope, and then finally in 1974 it was again renamed as *S. pneumoniae* (Musher, 2009).

S. pneumoniae is easily distinguished from other viridans-group streptococci on the basis of its colonial morphology. The colonies are greenish on blood agar and chocolate agar media (due to partial haemolysis), often mucoid due to the production of capsular polysaccharides and have a characteristic central depression, which is a result from the production of pneumococcal autolysin. In addition to the typical morphology, the basic methods in clinical microbiology laboratories to identify pneumococci are 1) optochin susceptibility and 2) bile solubility (Spellerberg and Brandt, 2015; Musher, 2009). Not all pneumococci are susceptible to optochin (Munoz et al., 1990; Kaijalainen et al., 2002). On the other hand, a recently described species, *S. pseudopneumoniae* is susceptible to optochin when grown in ambient air (Keith et al., 2006). The bile solubility tests identify *S. pneumoniae* with 99–100% sensitivity and 98–99% specificity (Kellogg et al., 2001). In recent years, the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) technique has emerged as an alternative for the identification of a wide range of bacteria, mycobacteria and fungi. The identification is based on the protein composition of the microbial cell. The ability of MALDI-TOF-MS methods to identify *S. pneumoniae* correctly is highly dependent on the platform used (Neville et al., 2011; deBel et al., 2010; Dubois et al., 2013; Branda et al., 2013; Kärpänöja et al., 2014; Harju et al., 2017).

S. pneumoniae colonizes the nasopharynx of healthy adults and children. Pneumococci are cultured from the upper airways in 5% to 10% of healthy adults and 20% to 40% of healthy children. The carriage rate has seasonal variation (highest in midwinter) and also population-based variation (ethnic and socio-economic differences). Pneumococci spread directly from a colonized person and cause infections like sinusitis and bronchitis in the respiratory tract and infections in the adjacent organs, e.g. otitis media. Haematogenous spreading may result in meningitis, pneumonia, primary bacteremia and other invasive infections (Musher, 2009). *S. pneumoniae* is among the most prevalent species that is cultured from middle ear fluids of children with acute otitis media and from patients with acute sinusitis (Wald, 2011; Ngo et al., 2016).

S. pneumoniae strains possess several virulence factors, of which the capsular polysaccharide is by far the most important. Antigenic differences in the capsule result in approximately 90 serotypes. A meta-analysis by Brueggeman and colleagues (2004) showed that pneumococcal serotypes and serogroups differ in invasiveness: serotypes 1, 5 and 7 were 60-fold more invasive than serotypes 3, 6A and 15. After introduction of the 7-valent and later the 13-valent pneumococcal vaccines in France, *S. pneumoniae* infections decreased in all age groups. The decrease was significant in invasive infections and in respiratory tract infections (Abat et al., 2015).

2.1.1.2 *Haemophilus influenzae*

Haemophilus influenzae belongs to the phylum Proteobacteria (Table 2).

Table 2. Taxonomy of *Haemophilus influenzae* (Brenner et al., 2005).

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Pasteurellales
Family	Pasteurellaceae
Genus	<i>Haemophilus</i>
Species	<i>Haemophilus influenzae</i>

The genus *Haemophilus* comprises eight bacterial species, which are associated with humans (*H. influenzae*, *H. aegyptius*, *H. ducreyi*, *H. pittmanniae*, *H. parainfluenzae*, *H. haemolyticus*, *H. parahaemolyticus*, and *H. paraprophaeolyticus*). They are gram-negative bacteria and the morphology ranges from small coccobacillary forms to filamentous rods. All species are facultative anaerobes. Nowadays, the genus is limited to human-associated species and no other natural hosts are known. The species of animal origin have been transferred to the genus *Pasteurella* (Ledeboer and Doern, 2015; Murphy 2009 a).

The genus name *Haemophilus* (blood-loving) refers to the characteristic that aerobic growth of bacteria in this genus requires two substances named as either X-factor or V-factor or both of them. X-factor is protoporphyrin IX, a metabolite in haemin biosynthesis. V-factor is a coenzyme composed of nicotinamide compounds (NAD or NADP) (Ledeboer and Doern, 2015).

To isolate *Haemophilus* species from clinical specimens, special attention has to be paid to the media used. Depending on the species, the medium has to provide the X-factor and/or V-factor. The X-factor is easily available for bacteria on traditional blood agar, but in order to release the V-factor, the blood cells have to be lysed (to prepare “chocolate–agar”). This factor can also be provided for the bacteria by cross-streaking *Staphylococcus aureus* or enterococci onto blood agar. *S. aureus* bacteria produce the V-factor and thus allow the growth of all *Haemophilus* species even on a common blood agar plate (satellite growth). Selective antibiotics such as bacitracin can be utilized to cultivate *Haemophilus* species from mixed flora of respiratory tract specimens (Ledeboer and Doern, 2015).

The first reports of *H. influenzae* were published by the German microbiologist Richard Pfeiffer in the 1890s (von Graevenitz, 2008). At that time it was claimed to be the agent that caused epidemic influenza. This theory turned out to be false. *H. influenzae* is the main species associated

with infections for the genus. Only one species, *H. ducreyi* (the causative agent of chancroid), is a primary pathogen, whereas all other species may be commensal as well (Ledebøer and Doern, 2015; Murphy 2009 a). *H. influenzae* was the first microbial agent apart from viruses, whose entire genome was sequenced (*H. influenzae* Rd; Fleischman et al., 1995).

Colonies of *H. influenzae* on chocolate agar are small (1–2 mm diameter), brownish, flat, smooth and semi opaque. Strains possessing a polysaccharide capsule are often mucoid. The species level identification is based on the typical colony morphology, a characteristic smell of growth (“mouse-nest smell”) and testing the growth factor requirements. A number of methods can be applied for the “factor” test: satellite growth-test to test V-factor dependency, paper-disk-test for X-dependent and V-dependent species and the porphyrin test for X-factor requirement. Four species induce haemolysis on bovine, horse or rabbit agar plates. The factor requirements and haemolysis reactions of different species in the genus *Haemophilus* are summarized in Table 3. *H. influenzae* and *H. aegyptius* can be differentiated using biochemical reactions, such as indole, urease and ornithine decarboxylase (Ledebøer and Doern, 2015; Munson et al., 2002). MALDI-TOF mass spectrometry methods identify *Haemophilus* species with good accuracy regardless of the platform used (Couturier et al., 2011; Powell et al., 2013).

Table 3. Principal differential characteristics of *Haemophilus*-species. Modified from Ledebøer and Doern (2015).

	Growth factor dependency		Haemolysis
	X	V	
<i>H. influenzae</i>	+	+	-
<i>H. aegyptius</i>	+	+	-
<i>H. haemolyticus</i>	+	+	+
<i>H. ducreyi</i>	+	-	-
<i>H. parainfluenzae</i>	-	+	-
<i>H. parahaemolyticus</i>	-	+	+
<i>H. pittmaniae</i>	-	+	+
<i>H. paraphrohaemolyticus</i>	-	+	+

H. influenzae frequently colonizes the upper airway, the lower respiratory tract and, rarely, the genital area of humans. 30 to 80% of humans carry noncapsulated *H. influenzae* in the nasopharynx. The carriage rate of type b-strains in vaccinated populations is less than 1%. The virulence and spectrum of diseases caused by *H. influenzae* is greatly dependent on the existence of capsular polysaccharide and its serotype in individual isolates. Non-typeable

(NT, which is equivalent to noncapsulated) strains are associated with RTI and invasive infections are mainly caused by encapsulated type b-strains. Other capsular types (a, c, d, e and f) and also certain NT isolates may occasionally be involved in invasive infections (Ledeboer and Doern, 2015; Murphy 2009 a). NT *H. influenzae* has been and continues to be a major pathogen along with *S. pneumoniae* and *Moraxella catarrhalis* in acute otitis media among children throughout the world (Sierra et al., 2011; Ngo et al., 2016; Sillanpää et al., 2016). There is some evidence, that *H. influenzae* is associated more frequently than other pathogens with recurrent and chronic otitis media (Casey et al., 2004; Hall-Stoodley et al., 2006). *H. influenzae* is also a common cause of other non-invasive infections such as sinusitis, exacerbation of chronic obstructive pulmonary disease (COPD), conjunctivitis and community acquired pneumonia. Invasive infections caused by *H. influenzae* type-b include purulent meningitis, epiglottitis, pneumonia, cellulitis, bacteremia and septic arthritis. These type-b diseases can be rapidly fatal without proper and prompt treatment, particularly in the cases of meningitis and epiglottitis. The conjugate-vaccines against type-b *H. influenzae* have decreased the numbers of invasive diseases among children in countries with wide-spread vaccination (Peltola, 2000). Among the low-vaccination countries and among certain populations (native Americans and native Alaskan children), in spite of covering immunizations, type-b *H. influenzae* still circulates. All invasive infections can also be caused by nontypeable and other serotype strains, but only occasionally. Special types of infections caused by nontypeable strains are neonatal and maternal sepsis, endometritis and ovarian abscesses. Usually biotype IV strains (closely related to *H. haemolyticus*) are involved in these cases (Murphy, 2009 a.).

2.1.1.3 *Moraxella catarrhalis*

The genus *Moraxella* belongs to a diverse group of miscellaneous gram-negative non-fermentative bacteria. The scientific classification is presented in Table 4.

Table 4. Taxonomy of *Moraxella catarrhalis* (Brenner et al., 2005).

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Kingdom	Bacteria
Phylum	Proteobacteria
Order	Pseudomonadales
Family	Moraxellaceae
Genus	<i>Moraxella</i>
Species	<i>Moraxella catarrhalis</i>

The morphology of this group varies widely as it includes species that exist as long rods and other species as small cocci. The common features of these bacteria are the lack of a fermentative metabolism and significantly better growth in aerobic than in an anaerobic atmosphere. Most species are catalase-positive, the oxidase reaction varies and they are non-motile or motile (Vanechoutte et al., 2015).

Since the first descriptions in 1882 by Seiffert and in 1896 by R. Pfeiffer *Moraxella catarrhalis* has undergone a number of nomenclatural and taxonomic changes. It was first named (in Germany) as *Mikrokokkus catarrhalis*, and later classified as belonging to the genus *Neisseria*. In 1970 a new genus *Branhamella* was established for the species but in the 1980s it was transferred to the genus *Moraxella*. There is still some debate about the “correct” classification of the species, however (Berk, 1990, Catlin, 1990; Enright and McKenzie, 1997).

The genus *Moraxella* includes ~20 species, of which *M. catarrhalis*, *M. osloensis*, *M. nonliquefaciens* and *M. lincolnii* are commensal in human upper respiratory airways. Occasionally all species of the genus are isolated from superficial and even invasive infections in humans, though *M. catarrhalis* is the most frequent. Colonies of *M. catarrhalis* grow well on blood agars and/or chocolate agars, they are small (1–3 mm), smooth and opaque. The characteristic pinkish colour develops after prolonged incubation (48 hours). The colonies are easily misidentified as *Neisseria* species by colony morphology after overnight incubation. However, the typical sliding of colonies across the agar surface, when pushed by a loop, is a specific feature for *M. catarrhalis*. In gram-staining, *M. catarrhalis* appears as a small gram-negative diplococcus. *M. catarrhalis* is strongly oxidase, catalase and DNase positive. The ability to hydrolyse ester-linked butyrate groups is an easy way to separate *M. catarrhalis* from members of the *Neisseria* group in clinical laboratories, and several commercial methods are available for this purpose (Vanechoutte et al., 2015; Murphy 2009 b; Speeleveld et al., 1994). MALDI-TOF mass spectrometry has shown good performance in identifying *M. catarrhalis* isolates (Matthew et al., 2015).

M. catarrhalis colonizes upper respiratory tract of humans. The rate of colonization is affected by age. Between 1 and 5% of healthy adults carry *M. catarrhalis* in the upper airways. Among children the colonization rate may be up to 100% among certain populations. Other factors may also be involved: regional factors, season, environmental conditions, hygiene, living conditions, genetic characteristics of the population and host factors. Pneumococcal vaccines have also changed the patterns of nasopharyngeal flora: non-vaccine *S. pneumoniae* strains, NT *H. influenzae* and *M. catarrhalis* have replaced the vaccine-type *S. pneumoniae* (Murphy 2009 b).

The clinical manifestations of *M. catarrhalis* infections are: otitis media in children, the exacerbation of COPD in adults, sinusitis, pneumonia in older adults and, occasionally, bacteremia. *M. catarrhalis* is one of the predominant causes of otitis media and bacterial sinusitis along with *S.*

pneumoniae and *H. influenzae*. It is detected in 15–20% of middle ear fluid (MEF) and sinus aspirate samples by culture and even more often (47%) with polymerase chain reaction (PCR) methods (Wald, 2011; Ngo et al. 2016; Sillanpää et al., 2016). *M. catarrhalis* is involved in mixed infections more often than other bacterial species and it is also found in children with a previous history of acute otitis media (Sillanpää et al., 2016). It also appears more often in young children (<12 months), and causes less spontaneous perforation of the tympanic membrane than other bacterial species (Kilpi et al., 2001; Broides et al., 2009). Immunodeficiency and other underlying diseases such as diabetes, malignancy and cardiopulmonary diseases are associated with *M. catarrhalis* bacteremia and pneumonia in adults (Murphy 2009 b).

2.1.2 Other respiratory pathogens

Other bacterial pathogens reported in MEF samples of children include: *Streptococcus pyogenes*, *Staphylococcus aureus*, *Chlamydia trachomatis*, *Pseudomonas aeruginosa* and members of the *Enterobacteriaceae* family. Additionally, bacteria with unclear significance in otitis media pathogenesis have been detected, such as *Alloiococcus otitidis* and *Turicella otitidis* among others (Ngo et al., 2016; Sillanpää et al., 2016). Viruses also play a significant role in children's otitis media, either alone or in combination with bacterial pathogens (Ruohola et al., 2006).

Several other bacterial species including anaerobic bacteria, staphylococci, *Enterobacteriaceae*, *Pseudomonas aeruginosa*, β -haemolytic streptococci, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae* are involved in other upper and lower respiratory tract infections such as bronchitis, pharyngitis, chronic sinusitis and pneumonia (Ruuskanen and Heikkinen, 2011; Korppi and Järvinen, 2011).

2.2 Antimicrobial resistance among respiratory pathogens

Penicillin/ampicillin with and without β -lactamase-inhibitor, macrolides, sulfamethoxazole-trimethoprim and tetracyclines antimicrobial agents have been most commonly used for RTI in Finland (Rautakorpi et al., 2001). The resistance rates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* for these are shown in figures 1, 2 and 3. The figures are modified from the database of the national FINRES-surveillance (FINRES, 2015), where susceptibility results from Finnish clinical microbiology laboratories are combined. Over 90% of *M. catarrhalis* produce β -lactamase and are therefore resistant to ampicillin (not shown in Fig. 3).

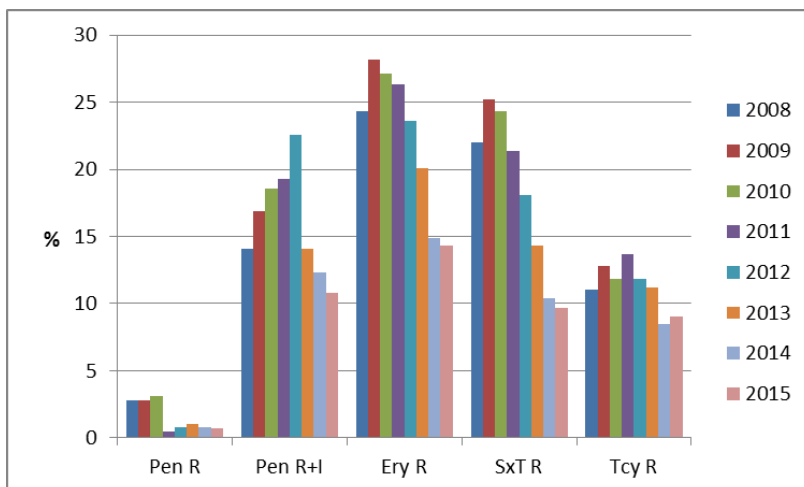


Fig.1. Antimicrobial resistance (% , y-axis) among *S. pneumoniae* in 2008–2015. R = resistant, I = intermediate; Pen = penicillin, Ery = erythromycin, SxT = sulfamethoxazole-trimethoprim, Tcy = tetracycline. Modified from FINRES (2015).

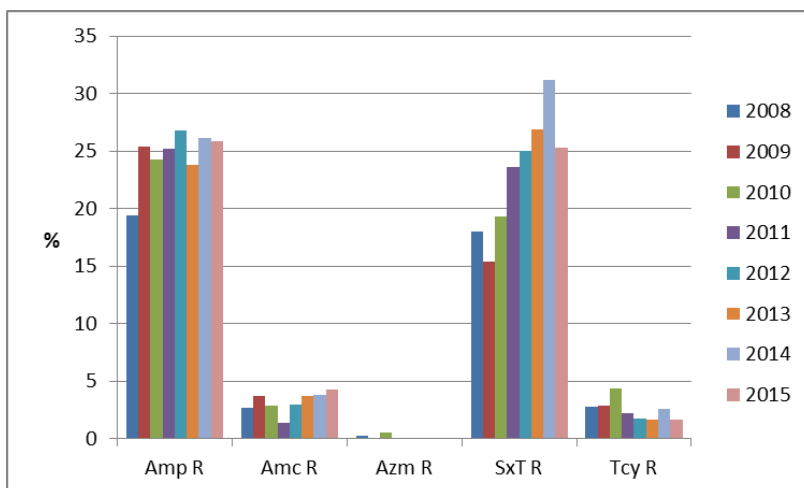


Fig. 2. Antimicrobial resistance (% , y-axis) among *H. influenzae* in Finland 2008–2015. R = resistant; Amp = ampicillin, Amc = amoxicillin-clavulanic acid, Azm = azithromycin, SxT = sulfamethoxazole-trimethoprim, Tcy = tetracycline. Modified from FINRES (2015).

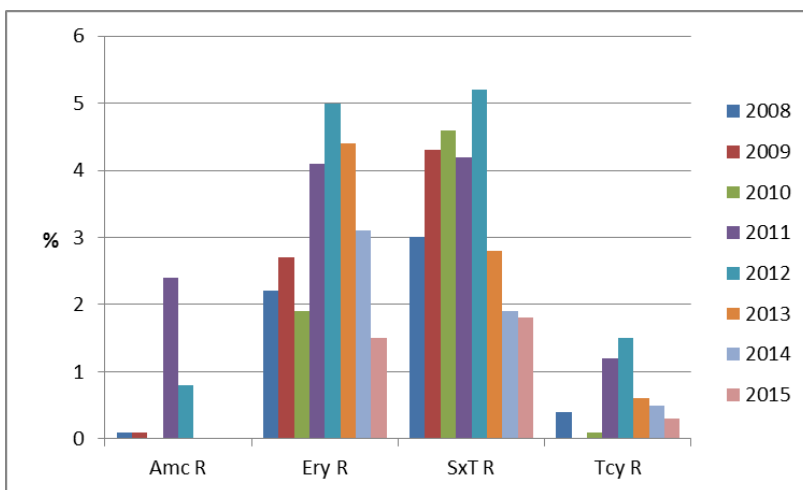


Fig. 3. Antimicrobial resistance (% , y-axis) among *M. catarrhalis* in Finland 2008–2015. R = resistant; Amc = amoxicillin-clavulanic acid, Ery = erythromycin, SxT = sulfamethoxazole-trimethoprim, Tcy = tetracycline. Modified from FINRES (2015).

2.2.1 β -lactams

The β -lactam ring is the common structure among all β -lactam antibiotics. The first and best characterized compound in this group is classical G-penicillin (Fig. 4), a natural β -lactam.

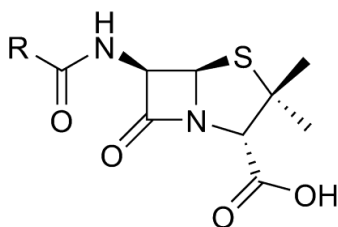


Fig. 4. The chemical structure of penicillin showing the four-membered β -lactam ring in the middle of the molecule. Modified from Walsh and Wencewicz (2016).

This group can be divided into two subgroups on the basis of chemical structures: penicillin-related and cephalosporin-related β -lactams. Additionally, non-classical β -lactams have been developed (monobactams and carbapenems). Benzylpenicillin and its derivative, ampicillin, are the primary β -lactams used for RTI. Benzylpenicillin is effective against gram-

positive bacteria; ampicillin is effective against gram-negative bacteria as well (Livermore and Williams, 1996).

All β -lactams are bactericidal antimicrobial agents and act by inhibiting the synthesis of peptidoglycan, which is the major component of the bacterial cell wall. Peptidoglycan is essential for bacteria, because it protects the cell contents from excessive osmotic pressure and also maintains the shape of the cell wall. The β -lactams bind to the D-alanyl - D-alanine transpeptidases and carboxypeptidases that mediate the cross-linking of adjacent sugar chains to form the peptidoglycan net-like structure. This binding is covalent and thereby blocks the building of peptidoglycan. Hence, these enzymes are called penicillin binding proteins (PBPs). Bacteria have different patterns of PBPs, which vary by molecular weight, function and affinity to different β -lactams. For instance, the PBP1a in gram-negative bacilli is a bifunctional transpeptidase/transglycosylase, whereas PBP4 is a carboxypeptidase. The affinity of different β -lactams for different PBPs also depends on the bacterial species (Livermore and Williams, 1996).

2.2.1.1 *Streptococcus pneumoniae*

S. pneumoniae is susceptible to many antibiotics including the β -lactams, macrolides, fluoroquinolones and vancomycin. However, acquired resistance has emerged in recent decades, especially against penicillin and macrolides. The resistance rates vary greatly in different parts of the world and the increase has been associated with an increased use of antibiotics in several studies (Musher, 2009; Riedel et al., 2007; Bergman et al., 2006; van de Sande-Bruinsma et al., 2008; Mera et al., 2006; Goossens et al., 2005). Dual- and multiresistance has also increased dramatically in some countries (Soares et al, 1993; Felmingham et al., 2005). Penicillin has traditionally been the drug of choice in both invasive and non-invasive pneumococcal infections. Since the 1970s, however, resistance to penicillin has increased in several countries and consequently penicillin is no longer the first line antibiotic used for invasive infections unless the susceptibility of the microorganism is known. The evaluation of changes in resistance is somewhat complicated, because the definitions of susceptibility and resistance have changed. Recently these definitions have been adjusted with respect to the site of infection (CLSI, 2015; EUCAST 2016). Penicillin non-susceptibility doubled (from 8 to 16%) among Finnish invasive pneumococcal isolates between 2002 and 2006 (Siira et al., 2009).

2.2.1.2 *Haemophilus influenzae*

Ampicillin and its analog, amoxicillin, are the primary β -lactams for superficial infections caused by NT *H. influenzae*. The drugs of choice for invasive infections that are usually caused by type b-strains are cefotaxime

and ceftriaxone (Murphy 2009 a). Ampicillin resistance has been moderate in the 2000s worldwide ranging from 1 to 27% in the USA and Europe (Morrissey, 2005; Perez-Tallero et al., 2010; Critchley et al., 2007; FINRES 2015). However, Asian countries including South-Korea and Japan had much higher resistance rates (58.5% and >50%; Bae et al., 2010; Sanbongi et al., 2006).

The outer membrane (OM) of gram negative bacteria typically complicates the penetration of β -lactam antibiotics into the host cell. However, the OM of *H. influenzae* is quite permeable to β -lactams, hence the MIC-values for ampicillin are lower than in other gram-negative bacteria such as *Enterobacteriaceae* (Livermore and Williams, 1996).

2.2.1.3 *Moraxella catarrhalis*

Since the first β -lactamase producing *M. catarrhalis* strains appeared in Finland at the end of the 1970s, their number increased very quickly to 95.9% in 1995 (Nissinen et al., 1995; Manninen et al., 1997). Ampicillin resistance has remained at a high level thereafter (FINRES, 2015). The proportion of β -lactamase-producers in other European countries has been in excess of 90%, in studies published in the 21st century, and virtually all isolates have been fully susceptible to amoxicillin-clavulanic acid, but resistance to cefaclor and cefuroxime was reported to be moderate to substantial in some countries (Schito et al., 2000; Morrissey et al., 2008; Gracia et al., 2008).

2.2.2 Macrolides and ketolides

Macrolides are a group of bacteriostatic antibiotics that share a common structure, which is formed by a 14 or 15 membered lactone ring (Leclercq and Courvalin, 2002). The chemical structure of erythromycin is shown in Fig. 5.

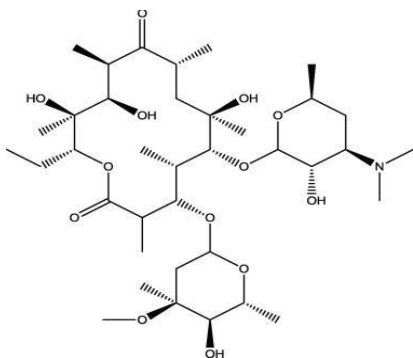


Fig. 5. The structure of erythromycin showing the 14 –membered lactone-ring. Modified from Walsh and Wencewicz (2016).

Erythromycin, clarithromycin and roxithromycin are the commercially available macrolides in Finland. In addition, one azalide antibiotic, azithromycin, and one ketolide antibiotic, telithromycin, are also commercially available. The term macrolide hereafter refers to the whole group of antibiotics that possess the lactone ring.

Macrolides act by blocking protein synthesis in the 23S rRNA subunit of the bacterial ribosomes. They bind near to the peptidyltransferase centre in domain V of the 23S rRNA subunit. The binding site is located in a tunnel, which is a channel for the growing peptide chain. Other major components in the tunnel are the ribosomal proteins L4 and L22. Erythromycin inhibits bacterial protein synthesis by blocking this tunnel (Leclercq and Courvalin, 2002).

2.2.2.1 *Streptococcus pneumoniae*

Erythromycin resistance increased among pneumococci in all parts of Finland from 1997 to 2002 and a significant association was found between erythromycin resistance and the consumption of azithromycin (Bergman et al., 2006). The increase has been reported separately among invasive isolates between 2000–2006 and ranged from 7% to 28% (Pihlajamäki et al., 2003; Siira et al., 2009). Resistance in non-invasive *S. pneumoniae* isolates rose to a peak in 2011 among children, after that resistance has decreased (FINRES, 2015). Erythromycin resistance has been especially high among penicillin-non-susceptible pneumococci (up to 62%) (Pihlajamäki, 2000; Kajjalainen et al., 2002). The 10 year surveillance in USA, UK, France, Italy and Spain reveals a similar trend for erythromycin and azithromycin but in Germany the increase was slower (Felmingham et al., 2005). The same study also reported a dramatic increase in the prevalence of penicillin-erythromycin co-resistance in France, Spain and USA. Two recent studies from Portugal and Spain show moderate increase in erythromycin resistance from 21.6% in 1996 to 25.3% in 2007 among all pneumococcal isolates (Simoes et al., 2011) and from 6.3% in 1997 to 19.4% in 2009 among invasive serotype 6C-isolates (Rolo et al., 2011). In England and Wales, on the other hand a sharp fall from 24% to 3% for erythromycin resistance among children's invasive isolates has been documented after the introduction of the heptavalent conjugate pneumococcal vaccine (PVC7) in 2006 (Henderson et al., 2010). Earlier, erythromycin resistance in pneumococci from blood cultures has been strongly associated with serotype 14, covered by the PVC7–vaccine in the UK (Birtles et al., 2004). In Canada, on the other hand resistance has increased especially among non-PVC7–serotypes (Wierzbowski et al., 2014).

2.2.2.2 *Haemophilus influenzae*

Numerous susceptibility surveillance studies including the macrolides have been conducted for *H. influenzae*. No indications of acquired resistance were detected for erythromycin in a Finnish study in 1995, though the authors stated the known fact that erythromycin has only a marginal effect on *H. influenzae* (Nissinen, et al., 1995). A point prevalence study from the USA, determined MIC-values for erythromycin, clarithromycin and azithromycin (Doern et al., 1997). In lack of breakpoints for erythromycin the results were compared in the light of MIC-values. The conclusion was that azithromycin was most active against *H. influenzae* (MIC₅₀/ MIC₉₀ –values 2/2 µg/ml). The corresponding values for erythromycin and clarithromycin were considerably higher (8/8 µg/ml and 8/16 µg/ml, respectively; Doern et al., 1997). European *H. influenzae* isolates from two periods (1997–1998 and 2002–2003) also showed higher MIC₅₀/MIC₉₀ values for clarithromycin (8/8 µg/ml) than for azithromycin (1/2) and telithromycin (2/2) (Fluit et al., 2005). The relatively low MIC values for, azithromycin especially, has allowed this antibiotic to be an option in the treatment of respiratory tract infections including those caused by *H. influenzae*. For example, in Finland 97% of macrolides were used for upper respiratory tract infections (URTI) and they were the most frequently used antibiotics by this indication (28%), followed by amoxicillin (25%) and doxycycline (20%) (Rautakorpi et al., 2001). Despite the good *in vitro* efficacy, the clinical efficacy of clarithromycin and azithromycin has been poor against *H. influenzae* (Dagan and Leibovitz, 2002).

2.2.2.3 *Moraxella catarrhalis*

Resistance to erythromycin among *M. catarrhalis* isolates has been moderate to low world-wide. The annual frequency of resistant isolates in Finland has varied between 1.9% and 5% (FINRES, 2015). In Estonia, 12% resistance to clarithromycin was reported in 2006 (Altraja et al., 2006). Another report of increased resistance comes from Italy, where between 5 to 10% of isolates had MIC-values for azithromycin and clarithromycin in the intermediate range (Tempera et al., 2010). A study from Japan, reported that the macrolides: erythromycin, clarithromycin, azithromycin and telithromycin showed strong respective activities against *M. catarrhalis* (Niki et al., 2011). Telithromycin was more effective against β -lactam-resistant *M. catarrhalis* than azithromycin (MIC₉₀ 0.06 vs. 0.12 µg/ml) (Leclercq, 2001). On the other hand, a global surveillance showed no substantial differences in the MIC-values of erythromycin and telithromycin for *M. catarrhalis* between the continents (Khan et al., 2010).

2.2.3 Sulfamethoxazole-trimethoprim

Sulfamethoxazole-trimethoprim (co-trimoxazole) is a combination of trimethoprim and sulfamethoxazole (see chemical structures in Fig. 6), and are usually used in the ratio 5:1 for treatment of infections (Walsh and Wencewicz, 2016).

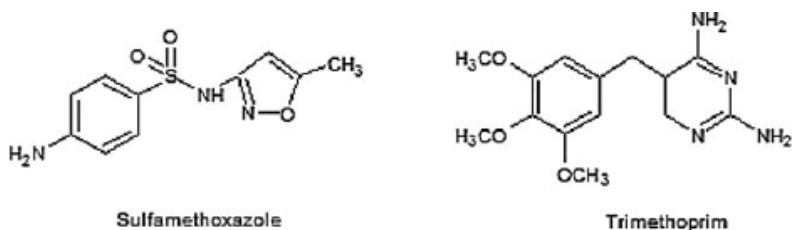


Fig. 6. Chemical structures of sulfamethoxazole and trimethoprim, the components of co-trimoxazole. Modified from Walsh and Wencewicz (2016).

The bactericidal effect is based on serial inhibition in the metabolic pathway of tetrahydrofolic acid (THFA) by the two components. THFA is essential for the synthesis of thymidine, methionine, glycine, adenine and guanine in both bacterial and mammalian cells. In contrast to human cells, bacteria are unable to absorb exogenous folates and are thus dependent of *de novo* synthesis of folates (except the genus *Enterococci*). The sulfonamide component inhibits the reduction of THFA in the presence of dihydrofolate-reductase (DHFR). Trimethoprim inhibits the action of DHFR (Eliopoulos and Moellering, 1996).

2.2.3.1 *Streptococcus pneumoniae*

Resistance to sulfamethoxazole-trimethoprim shows high geographical variation, even within the same country. Variation in resistance also depends on specimen type and resistance to other antimicrobial agents. In general sulfamethoxazole-trimethoprim resistance in Finland has increased from 4.5% in 1988–1990, among pneumococci (Nissinen et al., 1995) to nearly 30% in 2009 (FINRES, 2015), and thereafter resistance decreased. Substantial differences in resistance were reported in different regions of Finland (5.1% to 72.7%) in 1997 (Pihlajamäki et al., 2001). Total resistance in the USA was 23.5% in 2005–2006 (Chritchley et al., 2007). However, the resistance rate depended on penicillin susceptibility (5.1%) or non-susceptibility (37.3–73.1%) (Chritchley et al., 2007). A remarkably high incidence of resistance (81%) was reported from Taiwan in isolates collected

in a tertiary care hospital for the 1984–1997 period. Resistance was more common in respiratory isolates (>90%) than in blood culture isolates (72%) and it was also more common in penicillin resistant isolates (100%) than in penicillin susceptible isolates (84.6%) (Hsueh et al., 1999).

2.2.3.2 *Haemophilus influenzae*

Sulfamethoxazole-trimethoprim resistance in *H. influenzae* was fairly low in Finland <5% at the end of the 1980s (Nissinen et al., 1995), but has since gradually increased to the range of 25–30% annually (FINRES, 2015). Globally the incidence was at the same level in 1999–2000 (Hoban and Felmingham, 2002), and was the same in the USA in 2005–2006 (Critchley et al., 2007). Substantially, higher resistance rates have been reported in selected populations: in the Arab Emirates (37.3%) of patients with community acquired respiratory tract infections, in Taiwan from pediatric rhinosinusitis isolates (40%), in Zambia in carriage isolates obtained from HIV-infected children (38– 56%) and in India carriage isolates from healthy schoolchildren (67.3%) (Senok et al., 2007; Hsin et al., 2010; Mwenya et al., 2010; Jain et al., 2005).

2.2.3.3 *Moraxella catarrhalis*

Sulfamethoxazole-trimethoprim resistance among Finnish *M. catarrhalis* isolates has been low, only 2% in 2015 (FINRES, 2015). Earlier an increase from 0.5 to 14.5% over the 1995 to 1998 period in Finland has been reported (Manninen et al., 1997). Very low resistance rates have been reported in the 2000s in other countries: 0% in Canada, 2.2% globally from the PROTEKT surveillance (Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin) study (Bandet et al., 2014; Hoban and Felmingham, 2002). Recently, in Taiwan 18.5% resistance for *M. catarrhalis* isolates against sulfamethoxazole-trimethoprim was reported (Hsu et al., 2012).

2.2.4 Tetracyclines

The first tetracyclines were discovered in the 1940s and isolated from *Streptococcus aureofaciens* and *Streptomyces rimosus*. Tetracyclines are thus natural antimicrobial agents, although semisynthetic derivatives have been developed. The chemical structure of all tetracyclines and derivatives is based on a hydronaphthacene nucleus having four (the name “tetra” refers to four) fused rings (Fig. 7). Certain substitutions in this ring have resulted in semisynthetic compounds such as doxycycline and minocycline (Chopra and Roberts; 2001; Stratton 1996).

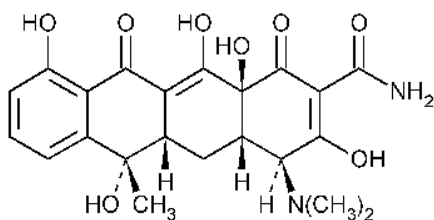


Fig.7. Structure of tetracycline molecule showing the four six-membered rings in the backbone of the molecule. Modified from Walsh Wencewicz (2016).

Tetracyclines exist in lipophilic form that can pass through the cell membrane and can also exist in hydrophilic form that diffuses easily throughout the cytoplasm. They use OmpF-porins and OmpC-porins to permeate the bacterial cell wall in gram-negative bacteria. Tetracyclines form magnesium-chelates to pass through these cation-dependent channels. The lipophilic form is also understood to cross the cell wall and the cytoplasmic membrane of gram-positive bacteria. Tetracyclines inhibit protein synthesis at the ribosomal level by preventing the codon-anticodon interaction between tRNA and mRNA. Binding of tetracyclines to the 30S ribosomal unit is reversible (Chopra and Roberts, 2001; Roberts, 2003; Stratton, 1996).

Tetracyclines are broad-spectrum bacteriostatic antibiotics that have activity against both gram-negative and gram-positive bacteria, intracellular organisms, such as chlamydiae, mycoplasmas and rickettsiae, and also certain eukaryotic organisms, such as *Plasmodium falciparum* and *Entamoeba histolytica*. RTI is, however, still the main indication for tetracyclines (Roberts 2003).

2.2.4.1 *Streptococcus pneumoniae*

Resistance to tetracycline has been moderate among *S. pneumoniae* in Finland. However, resistance has slightly increased: from 6.7% in 1995 to 9% in 2015 (Manninen et al., 1997; FINRES, 2015). Reports of resistance from other countries vary in the 2000s >90% in China (Hu et al., 2016), nearly 60% in Pakistan (Zafar et al., 2016), 20% in Turkey (Torumkuney et al., 2016) and 9% in the UK in (Blackburn et al., 2010). Dual-resistance and multiresistance has been reported in several studies, mostly with one or more of the following agents: penicillin, erythromycin and sulfamethoxazole-trimethoprim (Maraki et al., 2010; Lee et al., 2010; Vanhoof et al., 2010; Calatayud et al., 2010; FINRES, 2015).

2.2.4.2 *Haemophilus influenzae*

Tetracycline resistance in *H. influenzae* has been very low in Finland. Between 2008 and 2015 resistance has been <5% annually (FINRES, 2015). The PROTEKT-study reported 0.7% resistance for β -lactamase-negative isolates and 16.6% for β -lactamase-positive isolates globally in the 1999–2000 period (Hoban and Felmingham, 2002). *H. influenzae* strains from sputum specimens in the UK were highly susceptible (99%) to tetracycline over the 2007–2010 period (Blackburn et al., 2011). Very recent surveillance studies report higher resistance levels in the Middle-East and Far-East countries (Zafar et al., 2016; Torumkuney et al., 2016; Hu et al., 2016).

2.2.4.3 *Moraxella catarrhalis*

Very low prevalences (0–1.2%) of tetracycline resistance have been documented in Finland among *M. catarrhalis*-isolates over the 2008 to 2015 period (FINRES, 2015). Similarly, in the UK and Ireland 1999–2007 only 0.2% resistance was reported for 1999–2007 (Morrissey et al., 2008), and 1.1% resistance in Australia for the corresponding period (Pingault et al., 2010).

2.2.5 Resistance mechanisms

Resistance mechanisms reported in pneumococci, *H. influenzae* and *M. catarrhalis* to the four antimicrobial groups are summarised in Table 5 and are described in detail under heading numbers from 2.2.5.1 to 2.2.5.4 inclusive.

Table 5. Resistance mechanisms for β -lactams, macrolides, sulfamethoxazole-trimethoprim (=SxT) and tetracyclines in *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*.

Mechanism		
β -lactams	β-lactamases	
	<i>H. influenzae</i>	TEM1, ROB1
	<i>M. catarrhalis</i>	BRO1, BRO2
	Altered PBBs	
	<i>S. pneumoniae</i>	PBB1A, 2A, 2B, 2X
	<i>H. influenzae</i>	PBB3A, 3B
Macrolides	Ribosomal methylation	
	<i>S. pneumoniae</i>	<i>erm</i> (A), <i>erm</i> (TR)
	<i>H. influenzae</i>	<i>erm</i> (A), <i>erm</i> (B), <i>erm</i> (C), <i>erm</i> (F)
	Efflux pump	
	<i>S. pneumoniae</i>	<i>mef</i> (A), <i>mef</i> (E)
	<i>H. influenzae</i>	<i>mef</i> (A)
	Modified target site	
	<i>S. pneumoniae</i>	Domain V of 23S rRNA (A2058G, A2059G) Ribosomal proteins (L4, L22)
	<i>H. influenzae</i>	Domain V of 23S rRNA (A2058G, A2059G) Ribosomal proteins (L4, L22)
SxT	Modified enzymes (trimethoprim, DHFR)	
	<i>S. pneumoniae</i>	Modified <i>dhf</i>
	Overproduction of enzyme (trimethoprim, DHFR)	
	<i>H. influenzae</i>	ND
	Modified enzymes (sulfa, DHPS)	
	<i>S. pneumoniae</i>	<i>sulA</i> / <i>folP</i>
	<i>H. influenzae</i>	<i>sul2</i> , <i>folP</i>
Tetracyclines	Efflux	
	<i>H. influenzae</i>	<i>tet</i> (B)
	<i>M. catarrhalis</i>	<i>tet</i> (B)
	Ribosomal protection	
	<i>S. pneumoniae</i>	<i>tet</i> (M), <i>tet</i> (O)
	<i>H. influenzae</i>	<i>tet</i> (M)

2.2.5.1 β -lactams

Bacteria can become resistant to β -lactams by several different mechanisms, namely: altered PBPs, impermeability, production of β -lactamases and use of an alternative transpeptidase (Musher, 2009; Livermore and Williams, 1996).

In pneumococci, modifications in the PBPs 1A, 2A, 2B and 2X result in decreased affinity for the antibiotic and consequently decreased susceptibility (Linares et al., 2010). Low-level penicillin-resistance has been associated, especially with altered PBP2X, whereas PBP2B plays a vital role in clinically relevant resistance (Smith et al., 1993), although the final degree of resistance is presumably dependent on the collective action of multiple modified PBPs (Smith and Klugman, 1995).

Susceptibility to β -lactams in *H. influenzae* can be predicted by susceptibility to ampicillin and two mechanisms have been confirmed to mediate resistance: production of β -lactamase and/or altered PBPs (Tristram et al., 2007). The β -lactamase-mediated resistance is more common in Europe (Fluit et al., 2005) and USA (Heilman et al., 2005) whereas altered PBPs with low affinity to ampicillin have become more prevalent in Japan (Hasegawa et al., 2006). β -lactamase-mediated ampicillin resistance was first detected in *H. influenzae* in the 1970s; two types of β -lactamases, TEM-1 and ROB-1 are found in clinical isolates. Both are active against ampicillin and amoxicillin and are easily inhibited by β -lactamase inhibitors, such as clavulanic acid (Tristram et al., 2007). The PROTEKT study (1999–2003) reported the global distribution of these enzymes, which showed the predominance of TEM-1 over ROB-1 (93.7% vs. 4.6%); only in Mexico and USA did the proportion of ROB-1 exceeded 10%. In the same study a minor proportion (1.2%) of isolates were found to carry β -lactamases, but was negative for TEM-1 and ROB-1-genes in PCR. The susceptibility pattern, however, was similar, which suggested a novel type of β -lactamase or mutations in the previously described types (Farrell et al., 2005). ESBL β -lactamases have been transferred to *H. influenzae* by recombination techniques but this has not yet been detected in clinical isolates (Tristram et al., 2005). A recent study by Schaar et al. (2014) showed that β -lactamases of nontypeable *H. influenzae* isolates are present in outer membrane vesicles.

β -lactamase-negative, ampicillin resistant isolates (BLNAR) carry altered PBPs and show reduced susceptibility to ampicillin and ampicillin- β -lactamase-inhibitor combinations and also other β -lactams, particularly to cefaclor and cefuroxime. *H. influenzae* has eight PBPs (IA, IB, 2, 3A, 3B, 4, 5 and 6). PBP3A and 3B are encoded by the *ftsI* gene and modifications in these proteins have been confirmed to be responsible for the BLNAR-resistance (Tristram et al., 2007). In most surveillance studies of BLNAR, MIC values ≥ 2 $\mu\text{g/ml}$ for ampicillin has been applied for categorization (Doern et al., 1997; Fluit et al., 2005; Skaare et al., 2010). The study by Ubukata and colleagues (2001), however, showed that even isolates with ampicillin MIC values of 1 and 2 $\mu\text{g/ml}$, had mutations in *ftsI* genes, which resulted in decreased affinity of PBP3. There is also evidence, that BLNAR strains with high ampicillin MIC values (range 8-16 $\mu\text{g/ml}$) possess an additional resistance mechanism, AcrAR efflux pump (Kaczmarek et al., 2004). Yet, another mechanism behind high MIC values is β -lactamase enhanced BLNAR –resistance, that is an isolate carrying both β -lactamase and altered PBPs (named as BLPACR for β -lactamase positive amoxicillin-clavulanate resistant, Doern et al., 1997). The cloning of TEM-3, 4 and 5-type β -lactamase genes into *H. influenzae* showed that they alone did not elevate MIC values against 3rd generation cephalosporins. However, together with altered PBP3, isolates containing these β -lactamase genes became

resistant against cefotaxime. BLPACR-strains with TEM-1 type β -lactamases remained susceptible for cefotaxime (Bozdogan et al., 2006).

Two types of β -lactamases have been found in *M. catarrhalis*: BRO-1 and BRO-2 of which BRO-1 is the more common. Globally the BRO-1/BRO-2 ratio is 95%/5%, although adults are more likely to be colonized by the BRO-2 type strains (Khan et al., 2010). Both enzymes are encoded by chromosomal genes, are phenotypically similar, they differ from each other only by one amino acid, they are easily transformed from cell to cell and are inactivated by β -lactamase inhibitors. BRO-1 strains have higher MIC values for ampicillin, which is probably a matter of quantitative difference (Verduin et al., 2002.) A variant, named BRO-3 has also been reported. The substrate profile was identical with BRO-1 and BRO-2, but in isoelectric focusing the banding patterns were different (Christensen et al., 1991). This finding has not been confirmed in other studies. Recently, it was shown that outer membrane vesicles produced by *M. catarrhalis* also carry β -lactamases among other compounds such as virulence factors. This feature also protects other upper respiratory tract pathogens from amoxicillin-induced killing, although these pathogens may otherwise be susceptible (Schaar et al., 2011). A single report has been published of TEM- β -lactamase in *M. catarrhalis* (Robledano et al., 1987). There are also data from Europe about β -lactamase negative strains showing penicillin resistance (Berk et al., 1996). These isolates have not been studied further and the penicillin resistance mechanism is unknown.

2.2.5.2 Macrolides

Ribosomal modification by methylation was the first identified mechanism of erythromycin resistance. The *erm* gene codes the production of methylases, which add one or two methyl groups to the binding site of erythromycin at nucleotide A2058 (*E. coli* numbering) of 23S rRNA. This is a key binding site for the antibiotic (Walsh and Wencewicz, 2016). In pneumococci the *erm* gene is usually carried in transposons, which are easily exchanged even between different bacterial species. Today, several *erm* genes have been identified; and of these *erm*(B) is predominant in *S. pneumoniae* isolates in Finland (Rantala et al., 2005). The *erm*(A) subclass *erm*(TR) genes have also been detected in pneumococci, but to a lesser degree (Farrell et al., 2002; Syrogiannopoulos et al., 2001). Active efflux is another major erythromycin resistance mechanism in pneumococci. The genes responsible for this efflux are *mef*(A) and *mef*(E), which are transferred in transposons. These genes have 90% similarity in the nucleotide sequences and are considered variants of the same gene (Roberts, 2008; Leclercq and Courvalin, 2002). The resistance genes that confer macrolide resistance differ geographically: the efflux-genes have predominated, in Finland, Scotland, the USA and Germany (Rantala et al., 2005; Amegaza et al., 2002; Farrell et al., 2002; Bley et al.,

2011). The prevalences for *mef* and *erm*(B) in Canada are almost equal (Wierzbowski et al., 2007), whereas, in Russia, Korea and Spain *erm*(B) is more prevalent (Reinert et al., 2008; Bae and Lee, 2009; Calatayud et al., 2010). Individual isolates may have both *mef* and *erm*-genes (Farrell et al., 2002).

The first reports of macrolide-resistant pneumococci with no known mobile resistance genes were published in 2000, first after selection *in vitro* by macrolide passage (Tait-Kamradt et al., 2000 a) and later also in clinical isolates (Tait-Kamradt et al., 2000 b). Those studies reported mutations in domain V of the 23S rRNA (C2611A, C2611G, A2058G and A2059G, *E. coli* numbering) and the ribosomal protein L4. Later, several mutations in the 23S rRNA, L4 and also in ribosomal protein L22 have been reported which conferred macrolide resistance in pneumococci (Pihlajamäki et al., 2002; Farrell et al., 2003; Davies et al., 2005; Leclercq and Courvalin, 2002).

A study of clinical *H. influenzae*-isolates compiled in the Alexander-project (an international, multicentre, longitudinal surveillance study of antimicrobial susceptibility among common respiratory pathogens), revealed that almost all strains had an efflux-mechanism, which pumps clarithromycin and azithromycin out of the bacterial cells. This was shown even in isolates, which were classified as being susceptible on the basis of CLSI-breakpoints. Only in a minority of isolates with MIC values <0.25 µg/ml for azithromycin and <2 µg/ml for clarithromycin, well below the breakpoint values at that time (≤4 and ≤8, respectively), no indications of macrolide efflux was noted (Peric et al., 2003). The efflux was later shown to inhibit accumulation of telithromycin as well in *H. influenzae* -cells (Bogdanovich et al., 2006).

When 18 *H. influenzae* isolates were exposed to clarithromycin and azithromycin in a multistep resistance selection study (max. 50 days), >4-fold increases were measured in MIC values. None of these resistant strains had acquired the resistance genes *erm*(A), *erm*(B), *mef*(A/E) or *ere*(A) (an esterase conferring resistance to erythromycin), but 10/18 isolates had mutations in the respective macrolide binding site of the 23S rRNA or ribosomal proteins L4 and L22 (Clark et al., 2002). These findings were later confirmed in clinical isolates (Peric et al., 2003, Bogdanovich et al., 2006). A single mutation in the L22-ribosomal protein of a resistant mutant (substitution of arginine by proline at position 88, R88P) was not, however, able to confer high-level macrolide resistance, if the efflux was not present (Peric et al., 2004). Although the mobile macrolide resistance genes *erm*(A), *erm*(B), *mef*(A/E) or *ere*(A) were not detected in these studies, the *mef*-gene has been experimentally transferred to *H. influenzae* from a *S. pneumoniae* donor (Luna et al., 2000). The first proof of these mobile genes in clinical *H. influenzae* isolates were reported among cystic fibrosis (CF) patients. The first report included four isolates from a single patient with CF. The strains were isolated over a nine month period and showed increasing macrolide resistance. The 3rd and 4th isolates had high MIC values (>256 µg/ml) to

azithromycin and erythromycin and carried 2–4 copies of *erm*(B) and *mef*(A) genes (Ojo et al., 2006). Another study collected 106 azithromycin and/or erythromycin resistant or intermediately resistant strains in a placebo-controlled treatment study of children with CF. Half of all patients received long-term azithromycin therapy and the other half received placebo. All isolates carried one or more of the following macrolide resistance genes: *erm*(A), *erm*(B), *erm*(C), *erm*(F) and *mef*(A). Selected isolates were screened for L4 and L22-mutations, but these results were negative (Roberts et al., 2011).

The low prevalence of macrolide resistance among *M. catarrhalis* may be the reason why resistance mechanisms have not been studied in this microorganism. *Mef* genes have been found in other gram-negative clinical isolates, such as *Acinetobacter junii* and *Neisseria gonorrhoeae* and have been transferred by conjugation to *M. catarrhalis* (Luna et al., 2000). The conjugation was successful even, when the gram-positive species, *S. pneumoniae*, was used as a donor. That study, however, did not state the level of resistance in the recipient-isolates after conjugation. In the taxonomically closely related species *N. gonorrhoeae*, methylase genes and mutation in the 23S rRNA have also been reported (Roberts et al., 1999; Roberts, 2004; Ng et al., 2002).

2.2.5.3 Sulfamethoxazole-trimethoprim

Intrinsic resistance to sulfamethoxazole-trimethoprim appears mainly in enterococci, because those bacteria can incorporate folates into their cells. Several other bacterial species can absorb thymidine, but this does not contribute to resistance (Tegmark-Wisell et al., 2008). Modified DHFR is the most important mechanism to confer resistance to trimethoprim, and nowadays several different types of resistant DHFRs have been recognized in gram-negative bacteria (Kehrenberg and Schwarz, 2005; Grape et al., 2007; Roberts, 2002). Most of the DHFRs are spread in transposons or transferable gene cassettes (Huovinen et al., 1995). Gram-positive bacteria may also carry altered DHFRs, but hitherto only three resistant genes have been described. Resistance to sulfonamides is mediated by altered dihydropteroate synthases (DHPS). Several DHPS genes have been identified: *sulI* and *sulII* in gram-negative bacteria, *sulIII* in mycobacteria, *sulA* in pneumococci, and *folP* in several bacterial species (Huovinen et al., 1995; Fermer et al., 1997; Swedberg et al., 1998; Padaychee and Klugman, 1999; Roberts, 2002).

Sulfamethoxazole-trimethoprim resistance in *S. pneumoniae* was first reported in 1972 (Howe and Wilson, 1972). The individual MIC values of either component of the binary antimicrobial correlated with the MIC values of both combined in that study. However, in another study the MICs for the trimethoprim component correlated more strongly than the sulfamethoxazole component (Adrian and Klugman, 1997). Those authors also found more isolates that were resistant to the sulfamethoxazole but were

susceptible to the combination, than resistant to trimethoprim but susceptible to sulfamethoxazole-trimethoprim combination. This led to the conclusion that trimethoprim resistance is the essential factor in sulfamethoxazole-trimethoprim resistance. A single change in the DHFR (replacement of isoleucine-100 by leucine) due to mutation in the chromosomal *dhf* gene confers resistance to trimethoprim in pneumococci by inhibiting the binding site (Adrian and Klugman, 1997; Pikis et al., 1998; Marimon et al., 2006). Some additional mutations have been described, which may further decrease the susceptibility (Maskell et al., 2001).

The chromosomal *sulA*-gene, or *folP* as suggested by Haasum et al. (2001) encodes DHPS in *S. pneumoniae*. Several base insertions that lead to repetitions in the amino acid sequence of the DHPS enzyme have been detected in sulfonamide resistant isolates (Maskell et al., 1997; Schmitz et al., 2001). Apparently the insertions lead to an expansion and conformational changes in the region of the sulfonamide binding site, thus preventing its enzymatic effect (Padayachee and Klugman, 1999).

Only few published studies can be found about trimethoprim resistance mechanisms in *H. influenzae* and the data so far are somewhat speculative. de Groot and co-workers (1988) concluded first that trimethoprim resistance is due to overproduction of chromosomally located DHFR. In a further study (de Groot et al., 1991) they found structurally altered DHFRs with reduced affinity to trimethoprim in the resistant isolates. No plasmid-mediated resistance mechanisms were found in the study conducted by Powell (1991), who concluded that transposon encoded DHFRs, common in *Enterobacteriaceae*, had not spread to *H. influenzae* at that time (Powell, 1991). Sulfonamide resistance was investigated in clinical *H. influenzae* isolates of 24 highly resistant strains obtained from the UK and Kenya (Enne et al., 2002). The *sul1* was not detected, whereas *sul2* was present in 8/24 isolates and the remainder (16/24) had a 15 bp insertion in the chromosomal *folP* gene. This altered *folP* was transformed *H. influenzae* isolates into a susceptible recipient and the *folP* gene alone was able to increase the MIC value to sulfamethoxazole.

Currently, no studies have been published that demonstrate the mechanisms of resistance to sulfonamides or trimethoprim in *M. catarrhalis*. One study exists (Rådström et al., 1992), in which a sulfonamide susceptible *Neisseria meningitidis* isolate acquired resistance. It was suggested that the increase in resistance was acquired by the uptake of DNA from a sulfonamide resistant strain. The origin of the resistance mediating gene, however, remained unsolved in naturally resistant isolates. In this context the authors mentioned, that in *M. catarrhalis* altered *dphs*-genes were not found. *M. catarrhalis* is naturally resistant to trimethoprim due to trimethoprim-insensitive DHFR (Huovinen 1987).

2.2.5.4 Tetracyclines

Three mechanisms mediating tetracycline resistance in bacteria have been identified: efflux mediated resistance that exports tetracycline out of the cell, and ribosomal protection whereby binding of tetracycline molecule to the ribosome is prevented and enzymatic inactivation of tetracycline (found only in strict anaerobes). Today, 27 different genes that encode the efflux have been identified, a further 12 genes for the ribosomal protection mechanism and three genes for the enzymatic inactivation. In addition, one gene, with unknown mechanism is known, but does not seem to be related to either efflux or ribosomal protection (Roberts et al., 2012). The genes are named as *tet* (for tetracycline resistance) or *otr* (oxytetracycline resistance) (Roberts, 2005 and Roberts 2002).

Tetracycline resistance in pneumococci results from the acquisition of one of two resistance determinants, *tet(M)* or *tet(O)*. Both genes encode ribosomal protection proteins, which are able to dissociate tetracycline from the ribosome (Connell et al., 2003.) In both cases the release of tetracycline is strictly dependent on GTP (Burdett 1996; Trieber et al., 1998). The *tet(M)* gene is carried in conjugative transposons of the Tn916-family, which transfer readily between several gram-negative and gram-positive bacterial species. The Tn-like elements also contribute to multi-drug resistance by integrating into larger mobile genetic elements, which encode other antimicrobial resistance determinants (Rice, 1998).

Until recently tetracycline resistance in *H. influenzae* was associated only to the *tet(B)* gene, which encodes an efflux-mechanism. *tet(B)* is located on conjugative plasmids. Two other tetracycline-resistance mediating genes have been found in other *Haemophilus* species: *tet(M)* and *tet(K)* in *H. ducreyi* and *H. arophilus*. Both genes express ribosomal protection proteins, though only *tet(M)* does so in *H. ducreyi*. The *tet(M)* had been earlier transferred to *H. influenzae* *in vitro* by conjugation (Tristram et al., 2007.) In a recent study, *tet(M)* was found in clinical *H. influenzae* isolates (Soge and Roberts, 2011) Three strains isolated from CF patients, were resistant to erythromycin and tetracycline. All isolates carried both *tet(B)* and *tet(M)* genes and two of these isolates were able to transfer *tet(M)* to an *Enterococcus faecalis* recipient.

Four highly resistant *M. catarrhalis* strains have been characterized in the USA and in England in 1991 with respect to tetracycline resistance determinants. All carried *tet(B)* genes, which were non-transformable (Roberts et al., 1991).

2.3 Susceptibility testing methods

In vitro susceptibility testing in a clinical laboratory has two goals: to detect resistance in a particular isolate to antimicrobial agents, which are normally effective and to assure susceptibility to drugs of choice in the particular

infection (Jorgensen and Ferraro, 2009). Resistance and susceptibility may be confirmed by several methods. For the most part, laboratories use methods, which measure the ability of the antimicrobial agent to inhibit growth of the particular isolate, these include: the broth microdilution method (BD), the disk diffusion method (DD) and the gradient diffusion method. The result is reported, depending on the method, as quantitative (MIC) or qualitative (susceptible, S, intermediate, I or resistant, R). Another approach is to detect the actual mechanism behind the resistance (Jorgensen and Ferraro, 2009). For the first mentioned methodological approach there have been, historically, multiple guidelines with different breakpoints that divide bacteria into susceptible and resistant strains and attempts to harmonize this issue have been launched (Baquero, 1990, Kronvall et al., 2011). Different guidelines (standards) also include some methodological differences. The results of testing may be very different depending on the applied method and standard used. The differences also vary according to the antimicrobial agent, bacterial species and the genetic mechanism behind the resistance. For these reasons, the comparison of resistance data from different sources is complex (Cotter and Adley; 2001 Jones et al., 2004; Rodriguez-Martinez et al., 2011).

2.3.1 Guidelines for antimicrobial susceptibility testing

In order to harmonize antimicrobial breakpoints in Europe, the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) initiated the European Committee on Antimicrobial Susceptibility testing (EUCAST). Members of the EUCAST committees are chosen on the basis of their expertise in different areas of antimicrobial resistance and susceptibility testing. The work for setting breakpoints began in 2002 and the first breakpoints, quality control (qc) tables and the DD manual for susceptibility testing of bacteria were published in the end of 2009. All EUCAST information needed to perform and interpret antimicrobial susceptibility testing is freely available online (including the data behind the breakpoints, www.eucast.org; EUCAST, 2016). Finnish laboratories implemented the EUCAST method in 2011. The clinical MIC breakpoint is an MIC value that indicates probable therapeutic success. MIC breakpoints are defined on the basis of epidemiological cut-off values (ECOFFs, cut-off values), that distinguish wild-type strains from those carrying resistance mechanisms combined with pharmacodynamic and pharmacokinetic information. These breakpoints are calibrated in zone diameter values using an “MIC coloured histogram technique” (Fig. 8) (Kronvall et al., 2011; Wolfensberger et al., 2013; EUCAST, 2016).

The Clinical Laboratory Standards Institute (CLSI), previously The National Committee for Clinical Laboratory Standards, NCCLS) is a non-profit organization in USA, which produces standards and guidelines for

healthcare. Contrary to the EUCAST documents, the CLSI guidelines are not freely available. The Subcommittee for Antimicrobial Testing within this organization is composed of representatives from different fields: professional and governmental experts and representatives from pharmaceutical and diagnostic industry. The breakpoint setting in CLSI is based on correlating zone diameters to the corresponding MIC values (Fig. 9). It is assumed that the correlation between the inhibition zone size and MIC value is linear. MIC-values converted to logarithms are plotted against corresponding zone in millimeters (arithmetic scale). After regression analysis a straight regression line with best fit is drawn and from this line any break-points defined for MIC-values can be converted to analogous values on the mm-scale (Jorgensen and Turnidge, 2007).

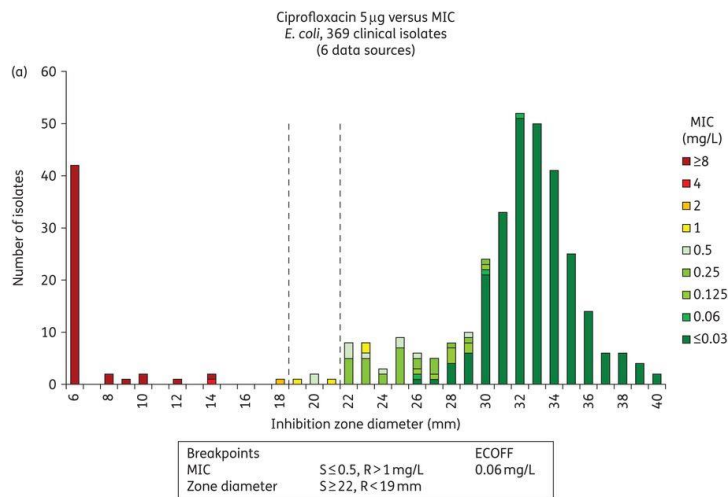


Fig. 8. An example of an MIC-coloured histogram used in the breakpoint setting by EUCAST (Kahlmeter, 2015). Reprinted with the kind permission of the copyright holder.

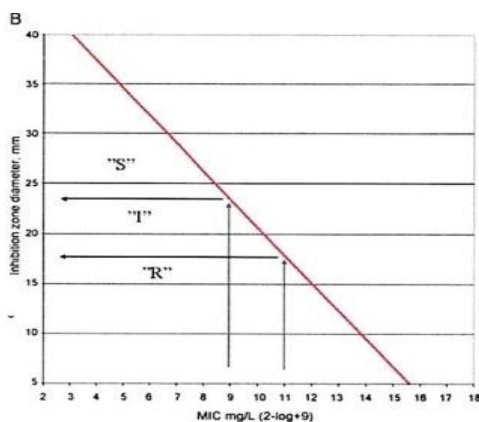


Fig.9. An example of the regression line –method for setting zone diameter breakpoints for tetracycline according to the CLSI –standard. MIC –breakpoints are marked with vertical arrows and corresponding zone diameters with horizontal arrows (Kronvall et al., 2011). Reprinted with the kind permission of the copyright holder.

Eleven other susceptibility testing standards were still being used in European clinical laboratories 2002: BSAC and Stokes in the United Kingdom, SFM in France, CRG in the Netherlands, DIN in Germany, Rosco in Belgium and Denmark, SRGA in Sweden, Czech in the Czech Republic, Mensura in Spain and a local guideline in Greece. Additionally national applications were used [e.g. FiRe (Finnish Study Group for Antimicrobial Resistance)–standard in Finland, which was adopted from the CLSI-guidelines] (Bruinsma et al., 2002).

2.3.2 Minimal inhibitory concentration

Either an agar dilution or BD (macro- or microdilution) method can be used to determine the MIC –value of an antimicrobial agent. These methods involve preparing two-fold dilutions of the antimicrobial agent in a liquid medium or agar medium, which supports growth of the isolate. The antibiotic tubes or plates are inoculated with a standardized bacterial suspension ($1-5 \times 10^5$ CFU/ml) and incubated overnight. The tubes or agar plates are examined with the naked eye or by automatic readers to define the lowest concentration of the agent e.g. 2 µg/ml, which prevents growth of the isolate (Fig. 10). This concentration represents the MIC value.

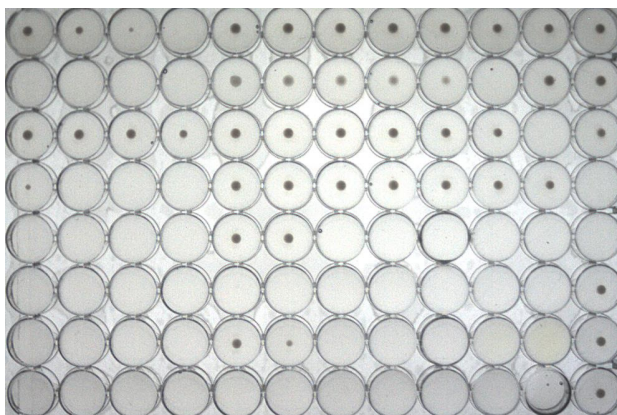


Fig.10. An example of determination of MIC values using BD - method on a 96–well microtiter platform (Sensititre®). The antimicrobial agents are in the wells in increasing concentrations. The spots indicate that the concentration is not able to inhibit the growth of the isolate. Photo is courtesy of Thermo Fisher Scientific. Copying is prohibited.

If the MIC-value is lower than the tissue concentration of the antibiotic at the infection site, then the isolate is considered susceptible (S) to the antimicrobial agent and the infection can be treated with normal dosage of the antibiotic. If the MIC value is correspondingly higher than its tissue concentration at the site, then the isolate is resistant (R) and normal dosage is not sufficient to achieve inhibition of the pathogen. Most guidelines include an interpretation area intermediate (I), which indicates that the isolate tolerates higher concentrations than normal and the inhibitory concentration cannot be achieved unless the drug is concentrated at the site of infection. The intermediate-category acts as a buffer between susceptible and resistant categories and is also meant to prevent false results due to the inaccuracy of the method itself. The broth and agar-dilution methods are too laborious and too slow to be used in a clinical microbiology laboratory setting. They are, however, the recommended reference methods for antimicrobial susceptibility testing (Amsterdam 1996; Jorgensen and Ferraro 2009; EUCAST 2016 and CLSI 2015).

Gradient diffusion methods, e.g. the E-test® (Epsilometer Test, bioMérieux, developed originally by Ab Biodisk), are widely used to define MIC values. The tests are based on agar diffusion. A plastic strip with a predefined, continuous gradient of the antimicrobial agent on one side and concentration scale on the other is placed with its antimicrobial impregnated-side face down onto an inoculated agar plate. The antimicrobial agent is rapidly released generating a stable gradient. After incubation an elliptical clear zone is formed and the intersection point, where the ellipse meets the strip indicates the MIC-value as read from the concentration scale (Fig. 11). The method has been evaluated for many pathogenic microbes,

including fastidious and anaerobic bacteria and yeasts, and it is considered a suitable alternative to the BD and agar dilution methods (Jorgensen et al., 1991). Other commercial applications similar to the E-test have been introduced recently are: M.I.C Evaluator™ by ThermoFisher Scientific and MIC Test Strip™ by Liofilchem.

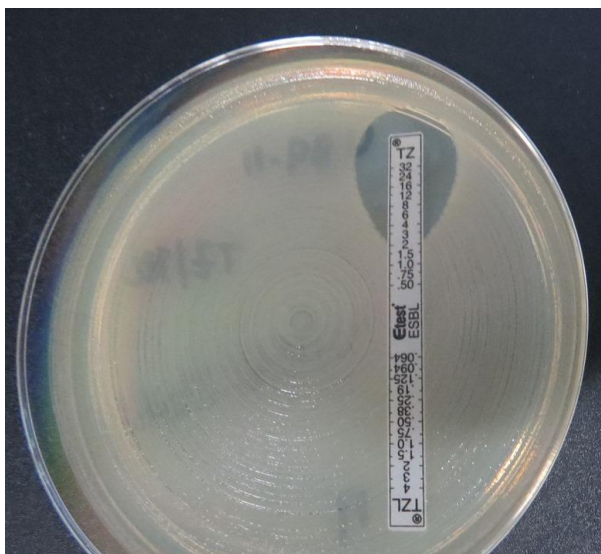


Fig. 11. Gradient diffusion test for *E. coli* to define MIC values (E-test®). TZ = ceftazidime (MIC 2 µg/ml), TZL = ceftazidime-clavulanic acid (MIC > 4 µg/ml). Photo Pauliina Kärpänöja.

2.3.3 The disk diffusion method

Being flexible and simple, the DD method (Fig. 12) is most widely used for susceptibility testing in clinical laboratories. The agar diffusion method was first used for measuring penicillin concentrations in plasma and in the 1940s it was adopted for susceptibility testing. For this purpose, filter paper disks impregnated with fixed antibiotic concentrations were developed. The first standard for disk diffusion susceptibility testing was published in 1966 and was named the Kirby-Bauer method after the authors (Bauer et al., 1966).

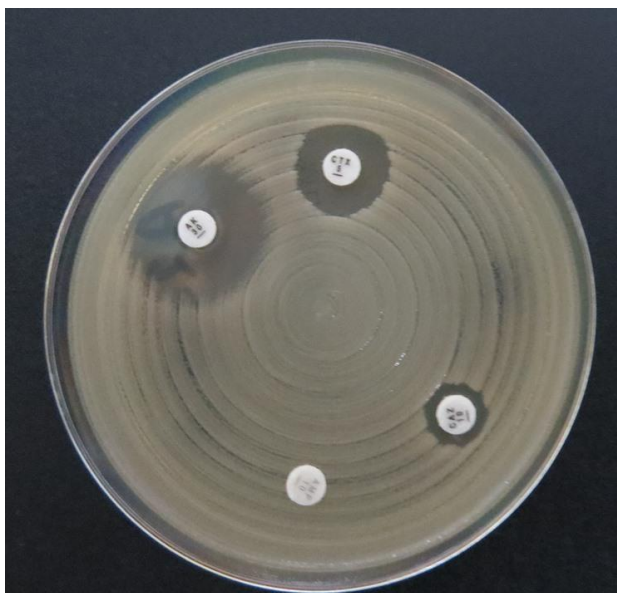


Fig.12. The disk diffusion (DD) test for *Klebsiella pneumoniae*. The isolate is resistant to ampicillin (AMP) and ceftazidime (CAZ), susceptible to cefotaxime (CTX) and amikacin (AK). Photo: Pauliina Kärpänoja.

Five years later, the World Health Organization (WHO) started a multicenter project to study the essential factors that influence the DD susceptibility testing. The report “International Collaborative Study” was published in 1971 (Ericsson et al., 1971). This method is different from the Kirby-Bauer method in inoculum density and was named according to the report of the study group as ICS method (Acar and Goldstein, 1996).

Several guidelines have been published since 1996. The common feature for all these is that the susceptibility result (S, I or R) is measured after incubation from the width of the inhibition zone that forms around the disk that was placed on an inoculated agar plate. The numerical value in millimeters is converted to the S/I/R scale using pre-set cut-off values.

The outcome of the DD method is affected by several factors. The composition of the agar medium is a crucial matter; Muller-Hinton (MH) agar is the most widely used media. However, it does not support the growth of all clinically relevant bacteria. The MH-medium can be enriched with blood to help the growth of fastidious bacteria. Other factors influencing the test results are: the inoculum density, timing of drug application, agar depth, incubation temperature, incubation time, growth characteristics of the test strain and the person to person variations in visual interpretation (Acar and Goldstein, 1996). The pre-set breakpoints, however, do not hold for all cases. Deviation occurs between different bacterial species and therefore most standards give breakpoints by species or species groups (Ericsson and

Sherris, 1971). Interlaboratory variation in determinations also occurs. However, careful standardization and quality assurance of the method can produce reliable and repeatable results (Nissinen et al., 1995; Manninen et al., 1995).

2.3.4 Automated instrument systems

Instrumentation of susceptibility testing may bring certain benefits: faster results, standardized techniques, the bias arising from visual reading can be avoided and the expert systems included in the software can help the interpretation of the results. There are a few studies about the effects of rapid susceptibility testing on different clinical and economical parameters. Two studies from USA, found significant benefits: the length of stay in hospital being shortened, the costs per patient were lower and the appropriate antibiotic therapy was initiated earlier (Doern et al., 1994; Barenfanger et al., 1999). A Dutch study found that, pathogen-directed antibiotic treatment was initiated earlier and total antibiotic use was reduced, when the susceptibility result was made available to clinicians earlier (Kerremans et al., 2008). In contrast, another hospital in the Netherlands, reported no impact in the variables assessed (mortality, morbidity and costs), even though the susceptibility results were reported significantly earlier (Bruins et al., 2005).

At least 12 automated instruments are available on the market for susceptibility testing, but three of them dominate: Phoenix® (Becton Dickinson), MicroScan® (Siemens) and Vitek2® (bioMérieux, Fig. 13). All three of these devices express susceptibility in MIC values and are supplied with software including an expert system. The MicroScan system is an incubator-reader system with manual inoculation, whereas the Phoenix and Vitek2-systems offer an additional automated inoculation (Winstanley and Courvalin, 2011; Jorgensen and Ferraro, 2009).



Fig.13. An example of automatic devices used for identification and susceptibility testing of bacteria (Vitek2[®], bioMérieux). Photo Pauliina Kärpänoja.

A cross-comparative study of the three methods for the susceptibility testing of *S. pneumoniae*, calculated the essential agreements (EA), which is the percentage of results within one dilution compared to the reference method and categorical agreements (CA), which is the interpretation within the same interpretation category as the reference method and compared them between all methods. The results were very similar for all instruments: Phoenix EA/CA 95.2%/99.3%, MicroScan 98.5%/99.5% and Vitek2 95%/98.8% for all antimicrobials tested. The reference method was BD microdilution according to the CLSI guideline. Major errors (ME) i.e. susceptible isolates reported as resistant were obtained most by the Vitek2 system (9.6%, Phoenix 9.3% and MicroScan 6.2%). The number of very major errors (VME) i.e., resistant isolate reported as susceptible was also highest with the Vitek2 (2.4%, Phoenix 0.3%, MicroScan 0%) (Mittman et al., 2009).

In another comparative study 347 clinical isolates (enteric gram-negative bacilli, non-enteric gram-negative bacilli, staphylococci, streptococci, enterococci and other gram-positive cocci) were identified. Then susceptibility tests were performed with these three compared instruments. The findings reported as ME/VME were 4/2 (MicroScan), 3/0 (Phoenix) and 1/3 (Vitek2). The total number of tests was 2723. The mean time taken to complete the antimicrobial susceptibility testing was shortest for the Vitek2-instrument (9 h), followed by Phoenix (12 h) and MicroScan (20 h) (Sellenriek et al., 2005).

Carbapenem resistance in *Enterobacter*-species was detected poorly by all three instruments. Sensitivity/specificity (%) readings were 100/0 for Phoenix, 82-85 /6-19 for MicroScan (two panels) and 74/38 for Vitek2. This

is partly explained by the fact that carbapenem resistance is often coupled with other resistance traits such as ESBL or AmpC combined with porin loss. There is considerable overlap in the MIC-values with these resistance mechanisms and consequently they poorly predict the carbapenemase resistance (Woodford et al., 2010).

2.3.5 Direct detection of resistance mechanisms

An old phenotypical method to detect resistance mechanism is the β -lactamase test. The most common procedures for the β -lactamase test are chromogenic cephalosporin test, acidimetric test and iodometric method. All these are based on the same principle: the end product of β -lactamase is visualized usually by colour change. For example, in the nitrocefin (chromogenic cephalosporin) test, the isolate studied is exposed to the nitrocefin disk. If β -lactamase is produced, then the amide bond in the beta-lactam ring will be hydrolyzed and a colour change occurs. This particular test detects most of the β -lactamases (ESBLs and carbapenemases excluded) and it is therefore widely used. However, for instance staphylococci require induction to express the β -lactamase production (Leitch and Boolayangor, 1992). In this case the cloverleaf test (or Modified Hodge's test, MHT) which requires an overnight incubation, can be used. The cloverleaf test detects the ability of the isolate studied to allow growth of a susceptible indicator-isolate in the presence of a β -lactam-antibiotic in case β -lactamase is produced (Kjällander and Myrbäck, 1964). Phenotypical tests are also used for detecting ESBLs, carbapenemases and AmpC-mediated resistance. The ESBL tests are based on the inhibitory effect of clavulanic acid and tazobactam. The isolate is incubated with a cephalosporin and cephalosporin-clavulanate combination. The forms and different sizes of the inhibitory zones (disk test) or differences in MIC-values (E-test) indicate ESBL-production (Steward et al., 2001; Cormican et al., 1996). Carbapenemases are screened using MHT or by demonstrating the inhibitory effect of different compounds (clavulanic acid, boronic acid or chelating agents such as EDTA). These methods, however, always require genetic confirmation (Queenan and Bush, 2007; Seah et al., 2011; Pasteran et al., 2009). AmpC mediated resistance has been screened by using ceftiofur as an indicator. Resistant isolates were confirmed phenotypically using the double-disk synergy method i.e. ceftiofur+oxacillin (Polsfuss et al., 2011).

2.3.6 Genetic and proteomic methods

Instead of determining phenotypic susceptibility in an isolate, detecting the resistance gene(s), may offer some of the following advantages for the laboratory and clinician: 1. the test can be performed directly on the sample without culturing; 2. phenotypic susceptibility tests will not detect the resistance, if the resistance gene is not expressed; 3. resistance may only be

manifested after prolonged incubation in slowly growing organisms, when phenotypic methods are used; 4. resistance can be determined even for non-cultivable bacteria and 5. culture of micro-organisms creates biohazard risks, which can be avoided by using direct genetic methods instead (Cockerill, 1999). However, there are also many limitations of this approach. The major drawback is that resistance to one antimicrobial agent may be due to several different mechanisms. Furthermore, behind one mechanism tens or even hundreds of different genes may be involved and all of these must be sought. An example of this is cephalosporin resistance in *Enterobacter* species. Compared to adding new probes to a PCR test, adding one antibiotic disk onto an agar-plate is far more flexible.

There are already a few commercial, molecular applications utilized in clinical microbiology. Methicillin resistant *Staphylococcus aureus* (MRSA) has been detected directly in different specimens (blood cultures, wounds, nasal swabs, and groin swabs) by several methods. For instance, the GenoQuick (HAIN Lifesciences), Xpert MRSA/SA (Cepheid), GenoType MRSA Direct (HAIN Lifesciences), Light Cycler (Roche) and GeneOhm Staph SR (Becton Dickinson) applications have been evaluated recently. The time required to get the result took 2–2.5 hours. Depending on the specimen type, sampling protocol and the test used, results have varied: sensitivity 57–98% and specificity 92.2–100% (Stamper et al., 2007; Scherlock et al., 2010; Bühlman et al., 2008; Wolk et al., 2009; Peterson et al., 2010). Other commercial applications include simultaneous identification and detection of rifampin resistance in *Mycobacterium tuberculosis* (Blakemore et al., 2010) and the probing of *vanA/vanB* genes (conferring vancomycin resistance) in enterococci (Marner et al., 2011). Additionally, several in-house, multiplex PCR applications have been introduced (Fluit et al., 2001).

Whole-genome sequencing (WGS) and next generation sequencing (NGS) techniques for the identification of antimicrobial resistance genes in bacterial whole genome data have been studied in recent years. High concordance 99.74% (Zankari et al., 2013) with phenotypic susceptibility testing has been achieved with several bacterial species and antimicrobial agents. Although the costs and complexity of sequencing techniques have recently declined, there is no consensus yet of the optimal bioinformatics method (Clausen et al., 2016). However, WGS and NGS are likely to become common in diagnostic microbiology in near future. Phenotypic susceptibility testing is still needed as a complementary method e.g. to identify novel resistance mechanisms (Dunne et al., 2012).

The MALDI-TOF MS method is based on protein analysis and is currently used in clinical laboratories for identifying bacteria and fungi. Recently other applications have been described, including the identification of antimicrobial resistance mechanisms. Carbapenemases in *Enterobacteriaceae* species have been detected by analyzing the degradation products of carbapenem using MALDI-TOF MS after incubating these beta-lactamases with the bacterial strain (Lasserre et al., 2015; Oviano et al., 2016).

Mather and colleagues (2016) differentiated accurately 98% of vancomycin intermediate (VISA) and vancomycin susceptible (VSSA) *Staphylococcus aureus* isolates by analyzing the presence or absence of any peak and the peak height of the MALDI-TOF spectra. Like the WGS approach, the MALDI-TOF MS cannot probably entirely replace phenotypic susceptibility tests, but has its place as an additional tool for selected purposes to detect resistance mechanisms (Hrabak et al., 2013).

2.4 Antimicrobial resistance and antimicrobial consumption

Penicillin was introduced into clinical medicine in 1944. In the period from 1946 to 1950 over 50% of originally susceptible *S. aureus* isolates worldwide had become resistant to penicillin. Plasmid mediated β -lactamases were the cause of this resistance. This is considered the first indication of antibiotic use being associated with antimicrobial resistance (Livermore and Williams, 1996). Since then, a number of studies have demonstrated the link between increased consumption and increased resistance at all ecological levels: in individual patients, hospital-settings, small communities, different geographical areas within one country, nationally and internationally. These studies cover different bacteria and antibiotic combinations. A positive association has been shown between consumption and resistance in carriers and also in clinical isolates (Arason et al., 1996; Bronzwater et al., 2002; Zervos et al., 2003; Bergman et al., 2004; McDougall et al., 2005; Malhotra-Kumar et al., 2007). Studies that found the opposite association i.e. a reduction in the use of antimicrobial agents was followed by a decrease in resistance also exist (Seppälä et al., 1997; Hsueh et al., 2006; Gottesman et al., 2009; Lee et al., 2010). Evidence has also accumulated of persistent resistance in the absence of a selective pressure; this has been shown with *Enterobacteriaceae* and streptomycin, with *E. coli* and sulfonamide and with *E. coli* and trimethoprim. It seems that genetic linkage of the index resistance to other genetic resistance elements (mobile transposons, gene cassettes) might partly explain this phenomenon (Chiew et al., 1998; Enne et al., 2001; Sundqvist et al., 2010). Bacteria have been traditionally assumed to become less fit, when they acquire resistance genes. If that is the case, then one might expect susceptible strains to predominate over time, once the use of the specific antimicrobial is reduced. However, there are studies showing that in some cases resistance populations remain in the population despite removing the selective pressure indicating that resistance does not necessarily reduce fitness of the bacteria (Melnyk et al., 2014; Holmes et al., 2016.)

2.4.1 The association between antimicrobial resistance and antimicrobial consumption with special reference to *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*

The association between antimicrobial resistance and consumption has been reported in numerous studies. Many of these studies used *S. pneumoniae* (Table 6) and show a positive association between one antimicrobial use and its resistance in the streptococcus. Resistance may also persist despite a decrease in antimicrobial use. Many studies also indicate, that consumption of one antimicrobial agent may also be associated to resistance to another antimicrobial class.

Similar studies on *H. influenzae* are less frequent (Table 7) but these still show a positive association between consumption and resistance.

Only a few studies deal with the development of resistance in *M. catarrhalis* with respect to antimicrobial consumption. Between 1978 and 1993 the number of β -lactamase positive *M. catarrhalis* obtained from middle-ear-samples of children showed a bimodal increase in Finland: from 0% in 1978 to 60% in 1983 and from 60% in 1988 to 80% in 1990. A 35-fold increase in the use of first and second-generation cephalosporins occurred at the same time with the second peak, and it had started a few years earlier (Nissinen et al., 1995). Macrolide consumption did not associate with the development of resistance in the study conducted by Cizman and colleagues (2001). In that study resistance rates were calculated from six years (1994-1999) and it was not until 1999 that the first macrolide resistant *M. catarrhalis* was observed.

Table 6. Association between antimicrobial use and resistance in *S. pneumoniae*.
↑ indicates increased, ↓ decreased and ↔ unchanged use or resistance.

Antimicrobial agent / consumption	Antimicrobial agent / resistance	Country / population / source of isolates	Ref.
Penicillin, low dose, long treatment	Risk factor for carriage of penicillin resistant isolates	France, children 3 - 6 years	Guillemot et al., 1998
Macrolides ↑	Erythromycin resistance ↑	Spain	Grazino et al., 2000
	High-level penicillin resistance ↑		
Oral cephalosporins ↑	High-level penicillin resistance ↑		
Total β-lactams ↑	Erythromycin resistance ↑		
Macrolides ↑	Macrolide resistance ↑	Finland	Pihlajamäki et al., 2001
Cephalosporins ↑	Macrolide resistance ↑		
Penicillins ↑	Macrolide resistance ↑		
Sulfamethoxazole-trimethoprim ↑	Sulfamethoxazole-trimethoprim resistance ↑		
Cephalosporins ↑	Sulfamethoxazole-trimethoprim resistance ↑		
Macrolides ↑	Macrolide resistance ↑	Slovenia, RTI ¹⁾	Cizman et al., 2001
Macrolides ↑	Macrolide resistance ↔	Slovenia, invasive isolates	
Azithromycin ↑	Macrolide resistance ↑	Finland	Bergman et al., 2006
Macrolides ↑	Macrolide resistance ↑		
Total β-lactams ↑	Penicillin non-susceptibility ↑	USA, blood culture isolates	Ruhe and Hasbun, 2003
Sulfonamides ↑	Penicillin non-susceptibility ↑		
Macrolides ↑	Penicillin non-susceptibility ↑		
Azithromycin ↑	Macrolide resistance ↑	Canada, national level	Karlowsky et al., 2009
Clarithromycin ↑	Macrolide resistance ↑		
Azithromycin ↑	Macrolide resistance ↔	Canada, regional level	
Clarithromycin ↑	Macrolide resistance ↔		
Macrolides ↓	Macrolide resistance ↔	Taiwan	Hsueh et al., 2006
Macrolides ↓	Macrolide resistance ↑	China	Chen et al., 2009
Ciprofloxacin ↑	Fluoroquinolone resistance ↑	Canada	Adam et al., 2009

1) RTI = respiratory tract isolates

Table 7. Association between antimicrobial use and resistance in *H. influenzae*.
 ↑ indicates increased, ↓ decreased and ↔ unchanged use or resistance.

Antimicrobial agent / consumption	Antimicrobial agent / resistance	Country / population	Ref.
Erythromycin ↑	Erythromycin resistance ↑	Sweden, children	Ringertz and Kronvall, 1987
Total β-lactams ↑	Amoxicillin resistance ↑	Scotland	Seaton et al., 2000
Macrolides ↑	Azithromycin resistance ↑	Slovenia	Citzman et al., 2001
Azithromycin, long-term treatment	Clarithromycin resistance ↑	Holland, CF ¹⁾ - patients	Phaff et al., 2006
Amoxicillin ↓	Amoxicillin resistance ↓	Spain	Garcia-Cobos et al., 2008
Sulfamethoxazole-trimethoprim ↓	Sulfamethoxazole-trimethoprim resistance ↓		

1) CF = cystic fibrosis

2.4.2 Resistance data in surveillance studies

A common view today is, that surveillance of antimicrobial resistance is needed at many different levels: locally, nationally and internationally. Longitudinal investigations, which enable monitoring trends over time, provide the most valuable data (Williams and Ryan, 1998; Morris and Masterton 2002; Halstead et al., 2004). Several national, continental and international surveillance programmes and networks are underway. However, as Neu and colleagues (1992) pointed out, there is a lack of standardization of the reported data and some data are fragmented and anecdotal.

Some specific projects/ of the surveillance programmes (e.g. the global Alexander-project, which focus on respiratory pathogens) rely on centralized susceptibility testing. The susceptibility results are considered reliable and comparable with this protocol (Felmingham et al., 2005). The global Antimicrobial Surveillance Program (SENTRY) and the Meropenem Yearly Susceptibility Test Information Collection (MYSTIC) utilize susceptibility testing results performed in several laboratories with common, well controlled methods (Rhomborg and Jones, 2009; Gales et al., 2011). The European Antimicrobial Resistance Surveillance Network, formerly the EARSS (EARSNet) by 2014 had collected resistance data from 29 EU/EEA countries starting from 2001. These data comprise routine susceptibility results provided by participating laboratories; the data are collected and compiled at the national level: such as the FiRe network (Finnish Study Group for Antimicrobial Resistance) in Finland (ECDC, 2015 a; Nissinen and Huovinen, 2000). Any variation in test methods between laboratories may

create a bias in such multilaboratory databases. The EUCAST organization aims, among other goals, at harmonizing break-points and standardizing methods to minimize this bias and to produce really comparable results between laboratories and countries (Kronvall et al., 2011).

A major defect in a surveillance report may be the inclusion of duplicate data (two or more identical isolates from one patient included). It was shown in the early 1980s that trimethoprim resistance rate of urinary tract isolates obtained from hospital patients decreased from 49.1% to 39.4% in the dataset after excluding duplicates. The decrease was smaller among outpatients (from 17.5 to 15.7 %; Huovinen, 1985). Horvat and colleagues (2003) showed that the rate of methicillin resistant *Staphylococcus aureus* (MRSA) was lower, when duplicate isolates were removed from the database. The difference was significant, when calculated for all hospital units and separately for non-intensive care units, but not significant in the intensive care unit (ICU). A Swedish study excluded duplicate isolates of *E. coli* and *S. aureus* from a 14-year consecutive database using different time cut-off times. When the cut-off was 90–365 days, resistance decreased, but when a 7–10 days cut-off was used, the effect was smaller among *E. coli*. Among *S. aureus* exclusion of duplicates resulted in small but systematic decrease in resistance. The authors, however, point out that one should not assume removing duplicate isolates always results in lower resistance rates (Sundqvist and Kahlmeter, 2007). There is no general agreement about what basis should be used to exclude duplicate isolates. For example, the WHONETsoftware (<http://www.who>) has different criteria that can be applied (first isolate only, most resistant isolate etc.; Morris and Masterton, 2002). The CLSI-guideline (M39-A3, 2009) stipulates that the first isolate of a given species per patient per analysis period, irrespective of body site, including its antimicrobial profile or other phenotypic characteristics must be used in the calculation of frequencies. This approach was supported by the study by Shannon and French (Shannon and French 2002 a).

The inclusion of screening samples in the resistance data may influence the outcome of resistance surveillance. Another study by Shannon and French (Shannon and French, 2002 b), showed that annual figures for methicillin resistance among *S. aureus* isolates differed 6-10% (with a limit of 365 days set for duplicate exclusion), depending on whether screening specimens were included or excluded. The authors also stated that inclusion of screening samples creates bias towards resistance mainly because patients are screened for colonization only with methicillin-resistant *S. aureus*, but not for methicillin-susceptible *S. aureus*.

The patient's age, sample type and patient type (hospitalized, outpatient) affect the resistance level. In France, the numbers of penicillin non-susceptible pneumococci were different in children and adults: blood cultures 27.8 and 32.5%, MEF-samples 60.2 and 27.5%, for children and adults respectively. Moreover, the decrease of penicillin-resistance in invasive infections was different in children at 46.4 to 29% compared to

adults from 43.4 to 32.7% (Kempf et al., 2011). A Finnish study of the invasive pneumococci characteristics between 2002 and 2006 reported that non-susceptibility for penicillin and erythromycin were highest among the age group 0–15 years (Siira et al., 2009).

Resistance data can be presented in several formats, the optimal format has not, however, been resolved among researchers. MIC-data are often summarized as MIC₅₀/MIC₉₀ i.e. the concentrations at which 50% and 90% of isolates are inhibited, and also as a range of MIC values (Duenas et al., 2011). This presentation model fits well for point-prevalence studies (Morris and Masterton, 2002). For continuing surveillance studies, such as the MYSTIC project, another presentation model has been introduced. The MIC results are expressed as the percentage of isolates inhibited at each drug-concentration. The MIC scale in this format should be large enough ($\geq 12 \log_2$ dilutions) to allow for minor drifts to be detected. The EARSNet surveillance collects the data as S/I/R results. However, the problems arising from this approach manifest as a lack of conformity, and no quantitative results available, which are acknowledged in the ECDC report (2015 a). Reporting of MIC values would be preferred. This is not a common practice in the participating laboratories (ECDC, 2015 a).

2.4.3 Consumption data

Studies on the association between antimicrobial use and resistance present antimicrobial consumption data mainly in two ways: defined daily doses (DDD), or prescription rates (Monnet et al., 2004).

The DDD is an internationally used unit for antimicrobial consumption, which was developed originally by the Nordic Council of Medicines in collaboration with WHO. The DDD is defined as the assumed average maintenance dose per day for a drug used for its main indication in adults. This value does not give exact figures of actual use of drugs, but an estimate of the overall consumption. An advantage of DDD is that is independent of price, currencies, package size and drug strength and is therefore considered a good measure to compare consumption between different populations (WHO Collaboration Centre, 2016). Outpatient antimicrobial use is often described as DDD/1000 inhabitants/day and is abbreviated in some studies as DID (Goossens et al., 2005). The drawback with measuring of antimicrobial consumption by DDD is that it does not provide information of the user (age, sex), the infection, the used dosage and whether the drug has been actually used.

The Drug Utilization Research Group (DURG) established by WHO in 1969 has recommended that international drug utilization studies classify drugs according to the ATC (Anatomic Therapeutic Chemical) system. In this system of classification, all drugs are categorized into 14 main groups according to the organ or system on which they act, in addition to their therapeutic, pharmacological and chemical properties (WHO Collaborating

Centre, 2016). Antibacterial agents are in the main class J, subgroup J01, Antibacterials for systemic use (Table 8).

Table 8. Antimicrobial classes according to the ATC/DDD-classification (adopted from WHO Collaborating Centre, 2015).

J	Anti-infectives for systemic use
J01	Antibacterials for systemic use
J01A	Tetracyclines
J01B	Amphenicols
J01C	β -lactam antibacterials, penicillins
J01D	Other β -lactam antibacterials
J01E	Sulphonamides and trimethoprim
J01F	Macrolides, lincosamides and streptogramins
J01G	Aminoglycoside antibacterials
J01M	Quinolone antibacterials
J01R	Combinations of antibacterials
J01X	Other antibacterials

In hospital settings, the DDD/100 patient admissions or patient days is a common way to present antimicrobial consumption instead of DDD/1000 inhabitants per day (Schön et al., 2011). The Slovenian Consumption Study Group compared hospital antibiotic use in five countries with three data presentation models. They noted that depending of the model used, the outcome was different. When consumption was expressed as DDD/100 bed-days, it was highest in Danish hospitals. If the model was DDD/100 admissions, hospitals in Holland used the most antibiotics. Finally, if the consumption was measured as DDDs/1000 inhabitants/day, it was highest in France (Cizman et al., 2011).

Several, mainly point-prevalence studies present antimicrobial use based on the number of prescriptions rather than DDD (or DID) figures (Steinman et al., 2009; Amadeo et al., 2010; McClean et al., 2011). The result may be different from the DDD data. Mölstad and colleagues (2002) compared antibiotic prescription rates between 13 European countries from 1994 to 1997. They found that the highest use of antibiotics occurred in Greece, followed by Spain and Belgium. Another study that covered almost the same period (from 1993 to 1997), reported sales of antibiotics in 15 European Union countries, showed that the consumption was highest in France, followed by Spain and Portugal (Cars et al., 2001). Their study used data

calculated as DDD/1000 inhabitants/day instead of the number of prescriptions. Moreover, a study by deWith and colleagues (deWith et al., 2006) revealed remarkable differences in the relative increase of antibiotic use in one hospital in Germany, depending on the data format. The relative increase of total antibiotic use was 81% as measured by DDD/100 patient days in medical wards, but only 48% when the PDD (prescribed daily doses)/100 patient days was used. One reason for this was the use of amoxicillin-clavulanic acid with different dosage from the DDD by the WHO index. One problem with consumption figures independent of the data format is undoubtedly the unknown share of antimicrobial agents used as self-medication. In some countries (even in Europe), antibiotics may be purchased legally without prescription from pharmacies (Grigoryan et al., 2006). For example, in Greece, 69-86% of pharmacists offered antibiotics without prescription for patients with high or low fever, and most of them offered even broad-spectrum agents (Contopoulos-Ionnadis et al., 2001).

2.4.4 Antimicrobial use in Europe and Finland

In Europe, total use of antimicrobial agents in 2014 was highest in Greece (34.0 DDD/1000 inhabitants/day) and lowest in the Netherlands (10.6 DDD/1000 inhabitants/day) (ECDC, 2015 b). The consumption figures from 30 European countries are shown in Figure 14.

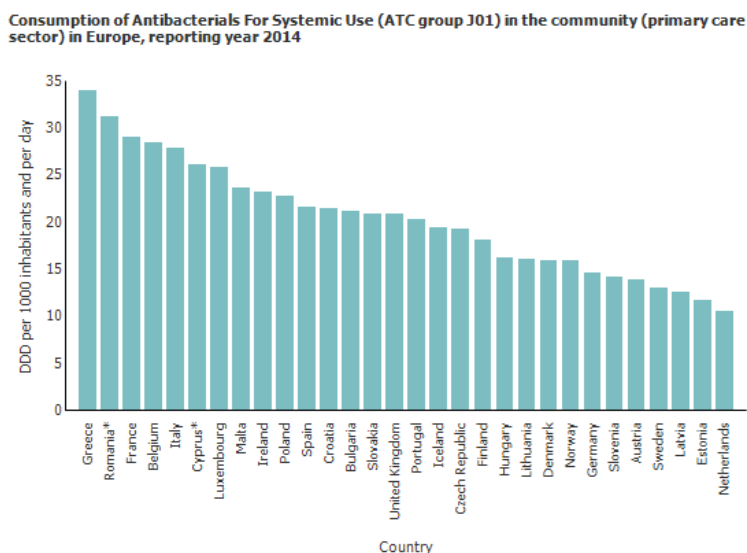


Fig.14. Total outpatient antibiotic use in 2014 in 30 European countries. Printed according to the ECDC Copyright and Limited Reproduction Notices (ECDC, 2015 b).

The use of antibiotics in Finland has slightly declined from 1990 to 2014 from 17.9 to 16.33 DDD/inhabitants/day, Fig. 15, Fimea 2016).

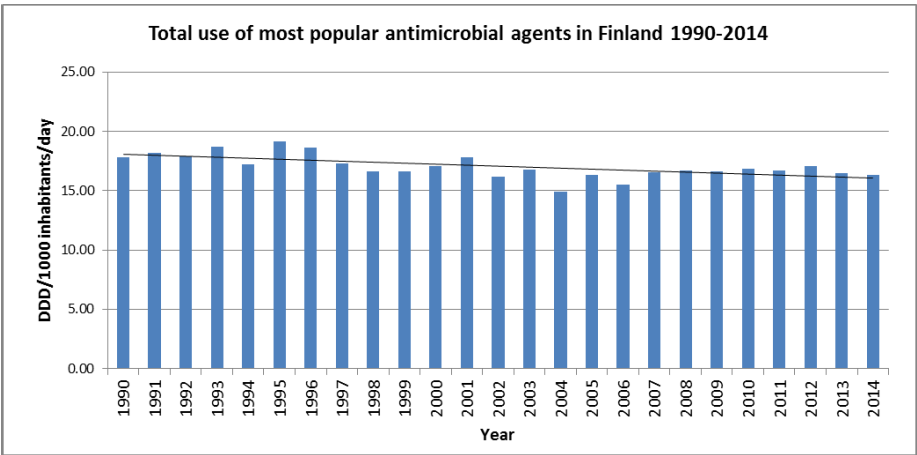


Fig. 15. Total use of the most popular antimicrobial agents in Finland 1990-2014 The consumption is expressed as DDD/1000 inhabitants/day (y-axis), modified from Fimea (www.fimea.fi, 2016).

Tetracyclines were the most commonly prescribed antibiotics in Finland in the 1990 to 2014 period, whereas the consumption of penicillin (phenoxymethylpenicillin) and sulfamethoxazole-trimethoprim declined. In contrast, the consumption of amoxicillin, amoxicillin-clavulanic acid and fluoroquinolone consumptions increased over the same period (Fig. 16, Fimea, 2016).

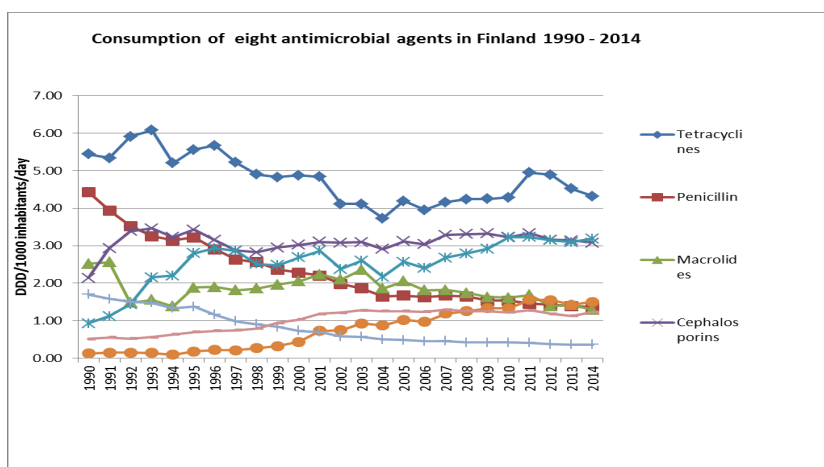


Fig.16. Consumption figures of the most used antimicrobial agents in Finland 1990-2014. The consumption is expressed as DDD/1000 inhabitants/day (y-axis). Modified from Fimea (www.fimea.fi, 2016).

Most (87.2%) of all the antimicrobial agents prescribed in Finland were used in the primary health care sector (ECDC, 2015, b).

3 AIMS OF THE STUDY

The Finnish susceptibility testing standard (FiRe-standard) was developed during the 1990s in order to standardize the methods for determining antimicrobial resistance in clinically important bacteria. The standardization project had two main goals: to produce reliable susceptibility results for clinicians and to achieve comparable resistance data between clinical microbiology laboratories in Finland. The general aim of the present study was to complete the standardization in Finland and to evaluate the results achieved by the standardization project. Prior studies have shown considerable variations in the methods used in Finnish laboratories for susceptibility testing and thus there has been a lack of uniformity in the resistance data collected nationally. Moreover, severe problems have been found in detecting unusual resistance, especially among the main respiratory pathogens: *Streptococcus pneumoniae* and *Haemophilus influenzae*. I have aimed in this thesis to evaluate how reliable the susceptibility results for these bacteria are in Finnish clinical microbiology laboratories and how resistance is affected by antimicrobial use.

The specific aims of this study were:

1. To validate the susceptibility testing method of *Haemophilus influenzae* for the national FiRe-standard with special reference to non- β -lactamase mediated ampicillin resistance (I)
2. To study the association between sulfamethoxazole-trimethoprim resistance and consumption in three common bacterial respiratory pathogens (II)
3. To assess the quality of Finnish resistance data of *Streptococcus pneumoniae* and *Haemophilus influenzae* by means of quality control results (III)
4. To evaluate an automated susceptibility testing method for *Streptococcus pneumoniae* (IV)
5. To identify the molecular mechanisms among macrolide resistant *Haemophilus influenzae* in Finland (unpublished).

4 MATERIALS AND METHODS

4.1 Bacterial strains (I, III, IV, unpublished)

Bacterial isolates used in study I, are summarized in Table 9. The isolates were used to validate the DD method for the susceptibility testing of *H. influenzae* for the national FiRe-standard.

Table 9. *H. influenzae*–isolates used in study I. UK NEQAS= United Kingdom National External Quality Assessment Services, ATCC = American Type Culture Collection. BLNAS = β -lactamase-negative, ampicillin susceptible, BLNAR = β -lactamase-negative, ampicillin resistant, ND = not determined.

Isolate/Source	Reference MIC values (mg/L)				
	Ampicillin	Amoxicillin-clavulanic acid	Cefaclor	Cefuroxime	β -lactamase
1 (BLNAS) UK NEQAS 1141	0.25	1	ND	ND	negative
2 (BLNAR) UK NEQAS 2550	8	16 - 32	ND	64	negative
3 (BLNAR) UK NEQAS 5270	2 - 4	2 - 4	ND	>4 – 12	negative
4 (BLNAS) ATCC49766	ND	ND	1 - 4	0.25 – 1	negative
5 (BLNAR) ATCC49247	2 - 8	2/1 – 16/8	12 - 32	3	negative

Phenotypical susceptibilities of the three UKNEQAS–originated isolates are based on the information from the quality control scheme organizer. Two reference laboratories (the Public Health Laboratory, Cambridge, England and the Quality Assurance Laboratory, UKNEQAS, Health Protect Agency, United Kingdom) have performed MIC testing for the isolates and they were categorized according to the BSAC standard. Reference MIC values for the ATCC collection strains were obtained from the CLSI standard of that time. *H. influenzae* isolate, ATCC49247, has been shown to carry a mutation in the *ftsI* gene that leads to altered PBP3 (GenBank accession number FM163678.1), (Skaare et al., 2010). The strains were selected for this study primarily on the basis of their BLNAS or BLNAR phenotypes.

Bacterial isolates used in study III are presented in Table 10. The isolates are the quality control isolates for susceptibility testing of *H. influenzae* and *S. pneumoniae* according to the FiRe standard.

Table 10. Bacterial strains used in study III and their reference susceptibilities. S = susceptible, R = resistant, I = intermediate, ATCC = American type culture collection.

Isolate	Antimicrobial agent	Expected result (mm)	Expected result (MIC, µg/ml)	SI/I/R
<i>S. pneumoniae</i>				
ATCC49619				
	ceftriaxone		0.03–0.12	S
	cefuroxime		0.25–1	S
	chloramphenicol	23-27	2–8	S
	clindamycin	19-25	0.03–0.12	S
	erythromycin	25-30	0.03–0.12	S
	oxacillin	8-12		R
	meropenem		0.06–0.25	S
	moxifloxacin	25-31	0.06–0.25	S
	penicillin		0.25–1	I
	tetracycline	27-31	0.12–0.5	S
	sulfamethoxazole-trimethoprim	20-28	0.12/2.4-1/19	S
	vancomycin	20-27	0.12–0.5	S
<i>H. influenzae</i>				
ATCC49247				
	ampicillin		2–8	R ¹⁾
	amoxicillin-clavulanic acid			R ¹⁾
	azithromycin	13-21	1–4	S
	tetracycline	27-31	0.12–0.5	I
	sulfamethoxazole-trimethoprim	24-32	0.03/0.59–0.25/4.75	S

The protocol for quality control (qc) followed that described in the FiRe-standard: when a new method is introduced, the qc should be performed daily for 30 days; after that the qc should be continually performed weekly and always, when new batches of agar-plates or disks are taken into use. Thereby, the minimum frequency can be calculated to be 52 times per year. The qc-results for these challenge strains were collected from 25 laboratories as inhibition zone diameters (mm) (DD tests) or MIC values (µg/ml).

S. pneumoniae isolates used in study IV are presented in Table 11 and their susceptibility rates are shown in Table 12. The isolates were identified

using the VITEK® MS MALDI-TOF method (bioMerieux S.A. 69280 Marcy l'Etoile, France).

Table 11. *S. pneumoniae* isolates used to evaluate susceptibility testing methods. PHSOTEY= Päijät-Häme Social and Health Care Group.

Isolate	Source ¹⁾	n
<i>S. pneumoniae</i>	Consecutive blood culture isolates (PHSOTEY)	183
<i>S. pneumoniae</i> , penicillin non-susceptible	Miscellaneous non-invasive specimens (PHSOTEY)	46

Table 12. Susceptibility of 229 pneumococcal isolates used in study IV by the broth dilution method (Sensititre®).

Antimicrobial agent 1)	S		I		R	
	n	%	n	%	n	%
Penicillin						
Non-meningitis breakpoints	153/229	66.8	69/229	30.1	7/229	3.1
Meningitis breakpoints	153/229	66.8	0/229	0.0	76/229	33.2
Cefotaxime	198/229	86.5	29/229	12.7	2/229	0.9
Ceftriaxone	195/229	85.1	32/229	14.0	2/229	0.9
Meropenem						
Non-meningitis breakpoints	229/229	100.0	0/229	0.0	0/229	0.0
Meningitis breakpoints	199/229	86.9	30/229	13.1	0/229	0.0
Moxifloxacin	228/229	99.6	0/229	0.0	1/229	0.4
Levofloxacin	229/229	100.0	0/229	0.0	0/229	0.0
Erythromycin	153/229	66.8	0/229	0.0	76/229	33.2
Tetracycline	192/229	83.8	1/229	0.4	36/229	15.7
Sulfmethoxazole-trimethoprim	160/229	69.9	8/229	3.5	61/229	26.6
Vancomycin	229/229	100.0	0/229	0.0	0/229	0.0
Linezolid	229/229	100.0	0/229	0.0	0/229	0.0

1) Breakpoints according to EUCAST 2016. The breakpoints for penicillin and meropenem are provided separately for non-meningitis and meningitis isolates. S = susceptible, I=Intermediate, R=resistant.

Table 13 shows three subsets of the *H. influenzae* isolates that were used to investigate the molecular mechanisms that conferred macrolide resistance (unpublished).

Table 13. *H. influenzae* isolates used for tracing macrolide resistance mechanisms.

Year	n	Source	Reference
1988 - 1991	204	MEF ¹⁾ , children < 6 years, six Finnish laboratories	Nissinen et al., 1995
1999 - 2000	379	Consecutive clinical samples, all age groups, seven Finnish laboratories	Unpublished
2006 - 2011	130	MEF ¹⁾ and NPS ²⁾ -samples from children with suspected otitis, two Finnish laboratories	Ruohola et al., 2013

1) MEF = middle ear fluid

2) NPS = nasopharyngeal secretion

4.2 Specimen collection and handling (I, III, IV, unpublished)

All isolates were subcultured once when necessary, frozen at -70°C in 20% skimmed milk as stock cultures and drawn onto TSAB (tryptone soya agar with sheep blood, *S. pneumoniae*) or chocolate agar (*H. influenzae*) plates before analysis.

4.3 Susceptibility testing methods (I, IV, unpublished)

The DD tests in study I were performed in 26 clinical microbiology laboratories in Finland (FiRe laboratories). The isolates (Table 6), HTM agar plates (Oxoid Basingstoke, Hampshire, UK) and 6 mm paper disks (Oxoid) were prepared and delivered to the laboratories centrally from the Antimicrobial Research Laboratory, National Public Health Institute, Turku, Finland (currently National Institute for Health and Welfare). The following antimicrobials and disk concentrations were tested: ampicillin 10 and 2 µg, amoxicillin-clavulanic acid 30 (20+10) and 3 (2+1) µg, tetracycline 30 µg and sulfamethoxazole-trimethoprim 25 (1:19) µg. The inoculum was adjusted to 0.5 on the McFarland scale and the inoculated plates were incubated for 16-18 hours at 35±2°C, 5% CO₂. Disk diffusion testing in study IV was performed on Muller-Hinton for fastidious bacteria (MH-F) agar plates (www.eucast.org), otherwise the testing was as described above.

The E-test® gradient strip (bioMérieux S.A. 69280 Marcy l'Etoile, France) was used in studies III and IV to define MIC-values. The inoculum was adjusted and plates were incubated as presented in the previous section.

Vitek2® (bioMérieux S.A. 69280 Marcy l'Etoile, France), an automated susceptibility testing system with the novel AST-GP74 card was evaluated in susceptibility testing on pneumococci (IV). The testing was performed according to the manufacturer's instructions. Briefly: the inoculum was

prepared from an overnight culture, adjusted to McFarland 0.5, and used to inoculate the susceptibility testing card. The cards were overlaid to the Vitek2 instrument for incubation and interpretation. Results of pneumococcal susceptibility tests were compared to the broth dilution method (BD, Sensititre®), performed in the EUCAST Development Laboratory (Växjö, Sweden).

The MIC values for azithromycin, clarithromycin and telithromycin were determined by the E-test (see method on the previous sections) in order to trace the macrolide resistance among *H. influenzae* (unpublished). Only azithromycin and clarithromycin were tested in the 2006–2011 period because telithromycin strip was not available then. Isolates resistant to any antibiotic (n=6) were selected for further investigations (EUCAST-breakpoints, www.eucast.org).

All methods were controlled by using *S. pneumoniae* ATCC49619 and *H. influenzae* ATCC49247 as the appropriate reference.

4.4 Resistance data (II)

The annual resistance data for sulfamethoxazole-trimethoprim among *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were obtained from FINRES reports between 1998 and 2004. FINRES reports (in 1998 FINMAP report) have been compiled from the routine susceptibility testing results from 21 to 27 (annual variation) Finnish laboratories. These laboratories are members of the FiRe network (www.finres.fi/) and they represent laboratories of the public healthcare service and laboratories serving the private healthcare sector. These laboratories are estimated to cover over 95% of all resistance data produced in Finland. The data are reported as numbers and percentages of resistant isolates of all isolates studied in the laboratory. If the number of tested isolates fell below 30/laboratory/year, the data were excluded. Regional (by central hospital districts, n=21) results were combined for the study (i.e. when several laboratories reported results for one central hospital district). These surveillance data are based on results of the DD method and in a few laboratories of the E-test® method. The DD tests were performed and interpreted according to the national FiRe-standard (paralleling the CLSI-guideline). The quality control programme included susceptibility testing of *S.pneumoniae* ATCC49619 and *H. influenzae* ATCC49247 as internal quality controls and proficiency testing programmes by Labquality (www.labquality.fi) and UKNEQAS (www.ukneqasmicro.org).

4.5 Antimicrobial consumption data (II)

The antimicrobial consumption data of sulfamethoxazole-trimethoprim (II) were obtained from Fimea (Finnish Medicines Agency, formerly Finnish National Agency of Medicines, www.fimea.fi/). These data are expressed as DDD per 1000 inhabitants per day. The consumption figures used in study II for comparing regional use to regional resistance are based on the sales from wholesalers to pharmacies (the use in hospitals is not included) on the assumption that this portion represents the use of these antibiotics in community health care. These regional consumption data were collected over a seven-year period (1997 to 2003 inclusive) from 21 central hospital districts.

4.6 Detection of macrolide resistance genes and mutations in *Haemophilus influenzae* (unpublished)

Six isolates showing decreased susceptibility to at least one of the following agents: clarithromycin, azithromycin and/or telithromycin, were tested for macrolide resistance genes and mutations known to confer macrolide resistance. *H. influenzae* colonies from solid media were suspended in water. The suspension was heated in a heating block at 95 to 100 ° C for 10 minutes. This crude extract of total DNA was used for PCR reactions. The presence of following genes was studied: *erm*(A), *erm*(TR), *erm*(B), *erm*(C), *erm*(F) and *mef*(A). The primers that were used for PCR and PCR conditions are listed in Table 11. Positive controls for the *erm* and *mef* genes as described previously (Pihlajamäki et al., 2002) and chromosomal DNA from a *Bacteroides fragilis* strain including *erm*(F) gene (kindly provided by Prof. Charlotta Enlund, Karolinska Institutet, Stockholm, Sweden) were included in the PCR reactions.

Mutations at positions 2058 and 2059 (*E. coli* numbering) of the genes coding for domain V of 23S rRNA were analysed using the pyrosequencing method after PCR. Haanperä and co-workers (2005) have previously described the procedure in detail (see Table 14 for primers and details of the PCR reactions).

Sequencing of the genes encoding for the ribosomal proteins L4 and L22 was performed using an ABI PRISM Big Dye Terminator Kit (Applied Biosystems, Foster City, California). Primers used for PCR and subsequent sequencing, as well as the PCR conditions, are also presented in Table 14.

The sequences were compared to *H. influenzae* Rd KW20 strain (NC 00907, Genome Sequence Data Base accession number L42023).

Table 14. Primers used for PCR and sequencing reactions and PCR conditions

Gene	Use	Direction	Primer sequence (5'–3')	PCR conditions
<i>erm(A)</i> ¹⁾	PCR	Forward Reverse	TCTAAAAAGCATGTAAAAGAA CTTCGATAGTTTATTAATATTAGT	10 min. at 95°C (one cycle), 1 min. at 94°C, 1 min. at 48°C, 1 min. at 72°C (30 cycles), 5min at 72°C
<i>erm(TR)</i> ²⁾	PCR	Forward Reverse	CTTGTGGAATGAGTCAACGG TTGTTCAATTGGATAATTTATC	10min. at 94°C (one cycle), 30 s at 94°C, 30 s at 51°C, 1 min. at 72°C (38 cycles)
<i>erm(B)</i> ²⁾	PCR	Forward Reverse	GAAAAGGTACTCAACCAATA AGTAACGGTACTTAAATTGTTTAC	10min. at 94°C (one cycle), 30 s at 94°C, 30 s at 51°C, 1 min. at 72°C (38 cycles))
<i>erm(C)</i> ¹⁾	PCR	Forward Reverse	TCAAAACATAATATAGATAAA GCTAATATTGTTTAAATCGTCAAT	10 min. at 95°C (one cycle), 1 min. at 94°C, 1 min. at 40°C, 1 min. at 72°C (40 cycles), 5 min at 72°C
<i>erm(F)</i> ³⁾	PCR	Forward Reverse	CGGGTCAGCACTTTACTATTG GGACCTACCTCATAGACAAG	10 min. at 94°C (one cycle), 30 s min. at 94°C, 30 s at 50°C, 2 min. at 72°C (35 cycles)
<i>mef(A)</i> ²⁾	PCR	Forward Reverse	CTATGACAGCCTCAATGCG ACCGATTCTATCAGCAAAG	10min. at 94°C (one cycle), 30 s at 94°C, 30 s at 51°C, 1 min. at 72°C (38 cycles)
23S rRNA ⁴⁾	PCR	Forward Reverse	TAAGGTAGCGAAATTCCTTGTCG (23SV_univF-1926) Bio-CGACCGCCCCAGTAACT (Bio_23SV_univR_2259)	10 min. at 95°C (one cycle), 15 s at 95°C, 15 s at 61 °C, 30 s at 72 °C (35 cycles)
Domain V of 23SrRNA	Pyrosequencing		CCGCGGCTAGACGG (23SV_Hinf_seq)	
L4 ⁵⁾	PCR	Forward Reverse	TTAAGCCGGCAGTTAAAGC CACTTAGCAAACGTTCTTG	10 min. at 95 °C (one cycle), 30 s at 94 °C, 30 s at 53 °C, 45 s at 72 °C (35 cycles) 7 min at 72°C
L22 ⁵⁾	PCR	Forward Reverse	CGGCAGATAAGAAAGCTAAG TGGATGTACTTTTGACCC	10 min. at 95 °C (one cycle), 30 s at 94 °C, 30 s at 53 °C, 45 s at 72 °C (35 cycles) 7 min at 72°C

- 1) Sutcliffe et al., 1996
- 2) Figueira et al., 2004
- 3) Roberts et al., 1999
- 4) Haanperä et al., 2005
- 5) Peric et al., 2005

4.7 Statistical methods (I, II, III, IV)

Whonet 5.3 software (<http://www.who.int/drugresistance/whonetsoftware/>) was used to store the results (I, III), for calculating the susceptibilities (I, III) and for drawing the histograms (I). SPSS for Windows statistical software

was used to calculate the means and standard deviations (SD) sensitivities, specificities and ranges (I).

In study II, a linear mixed model for repeated measures was used to model the association between regional resistance and regional antimicrobial consumption of the previous year. The resistance rates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in the 21 central hospital districts were compared separately to local consumption figures of sulfamethoxazole-trimethoprim. The percentage of resistant strains was taken as the dependent variable whereas the antimicrobial consumption and time were the explanatory variables. A random effects model with time and consumption as a fixed effect and the intercept representing the random effect was fitted. Mixed models were fitted using the Proc Mixed in the SAS System for Windows version 8.02. The level of statistical significance was set at p-values below 0.05.

The CA, EA, false susceptible rate (FSR), numbers of minor errors (mE), ME and VME were calculated according to the Food and Drug Administration (FDA) guidelines (2009) and previous reports (Skaare et al., 2015).

5 RESULTS

5.1 Susceptibility of *Haemophilus influenzae* (I, unpublished)

5.1.1 Validation of the disk diffusion method

Study I was undertaken to validate the disk diffusion susceptibility testing method of *H. influenzae* for the FiRe standard. The 26 laboratories participating in this study produced a total of 128 results (mm) for ampicillin 10 µg (Amp 10) disks and 129 results for ampicillin 2 µg (Amc 2), amoxicillin-clavulanic acid 30 µg (Amc 30) and amoxicillin-clavulanic acid 3 µg (Amc 3) disks. Interpretation of the results was based on the NCCLS breakpoints (currently the CLSI) breakpoints applied in 2004 and on the suggested breakpoints to lower concentration ampicillin according to Zerva (1996). These same breakpoints were used to lower concentration amoxicillin-clavulanic acid, as well. The results of each isolate for these two antimicrobial agents are presented in Table 15. Resistant and intermediate categories were combined for the analysis. Isolates 3 and 5, which were defined as BLNAR strains, were categorized more accurately with the low concentration disks. The result was not as evident for isolate 2 (BLNAR). False resistant results were provided equally for isolates 1 and 4, which were defined as BLNAS strains.

The calculated sensitivities and specificities for each test (disk) are summarized in Table 16. The sensitivity to detect the BLNAR resistance for both antibiotics was better when low concentration disks were used. Specificities were as good (ampicillin) or slightly better with the high concentration disks (amoxicillin-clavulanic acid).

Table 15. Results of ampicillin and amoxicillin-clavulanic acid disk diffusion susceptibility tests obtained from 26 laboratories. BLNAS = β -lactamasenegative, ampicillin susceptible, BLNAR = β -lactamasenegative ampicillin resistant. UKNEQAS = United Kingdom National External Quality Assurance Services. ATCC = American type culture collection.

Isolate	Interpretations			Inhibition zone diameters		
	Disk	S %	R+I %	Mean	(SD)	Range
1 (BLNAS), UKNEQAS 1141	Amp 10	81	19	25	(± 5)	16–38
	Amp 2	88	12	21	(± 5)	06–30
	Amc 30	96	4	25	(± 5)	16–36
	Amc 3	80	20	19	(± 4)	09–26
2 (BLNAR), UKNEQAS 2550	Amp 10	28	72	19	(± 6)	06–28
	Amp 2	24	76	13	(± 5)	06–23
	Amc 30	36	64	17	(± 6)	06–28
	Amc 3	24	76	10	(± 6)	06–24
3 (BLNAR), UKNEQAS 5270	Amp 10	38	62	20	(± 4)	13–30
	Amp 2	0	100	10	(± 2)	06–14
	Amc 30	65	35	29	(± 4)	15–31
	Amc 3	4	96	12	(± 2)	06–17
4 (BLNAS), ATCC49766	Amp 10	96	4	29	(± 4)	21–36
	Amp 2	92	8	23	(± 4)	14–30
	Amc 30	96	4	28	(± 5)	18–36
	Amc 3	92	8	22	(± 3)	14–28
5 (BLNAR), ATCC49247	Amp 10	20	80	20	(± 3)	11–25
	Amp 2	0	100	10	(± 2)	06–12
	Amc 30	65	35	20	(± 4)	11–31
	Amc 3	0	100	11	(± 2)	06–15

Table 16. Sensitivity and specificity values for four tests (disks) to detect BLNAR resistance in *H. influenzae*. Amp 10 = ampicillin 10 µg, Amp 2 = ampicillin 2 µg, Amc 30 = amoxicillin-clavulanic acid 30 µg, Amc 3 = amoxicillin-clavulanic acid 3 µg.

Test (disk µg)	N	Sensitivity (%)	Specificity (%)
Amp 10	128	71	88
Amp 2	129	92	90
Amc 30	129	44	96
Amc 3	129	91	87

In addition to ampicillin and amoxicillin-clavulanic acid, the performance of the DD method for tetracycline and sulfamethoxazole-trimethoprim susceptibility testing was evaluated (unpublished). Zone diameter values provided by laboratories were combined (sulfamethoxazole-trimethoprim n=97, tetracycline n=84) to create a histogram. The expected susceptibility distribution (S/I/R) for tetracycline was 30/0/60% and for sulfamethoxazole-trimethoprim 75/0/25% based on the information from UK NEQAS and ATCC (Fig. 17 and 18).

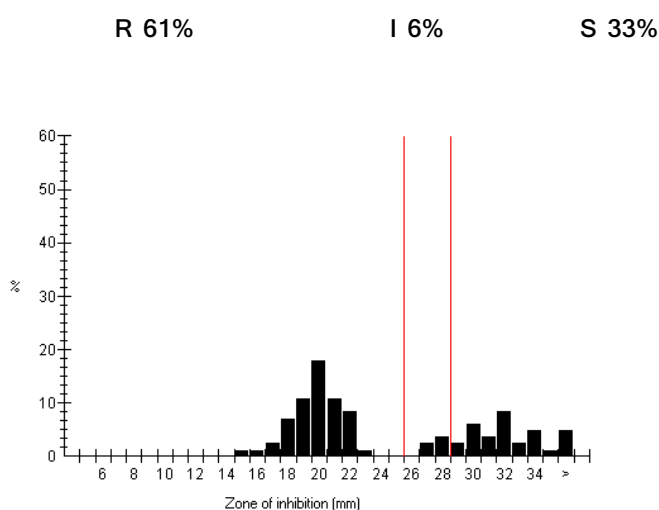


Fig.17. Distribution of inhibition zone diameters (mm) from 84 tetracycline disk (30 µg) diffusion tests. The vertical red lines indicate breakpoints defined by the NCCLS in 2004.

R 23%

S 77%

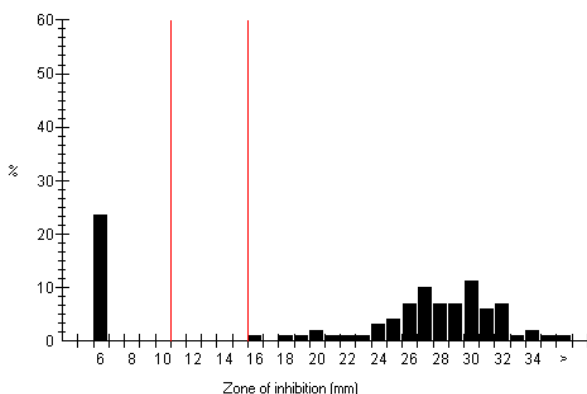


Fig.18. Distribution of inhibition zone diameters (mm) from 97 sulfamethoxazole-trimethoprim disk (25 µg) diffusion tests. The vertical red lines indicate breakpoints defined by NCCLS in 2004.

5.1.2 Macrolide resistance mechanisms in *Haemophilus influenzae* (unpublished)

A total of 6 out of 713 isolates tested were identified as being resistant to at least one macrolide (Table 17). One isolate (Hi66) was resistant only to azithromycin and three isolates only to telithromycin (Hi98, 212 and 218). Two isolates (Hi286 and Hi593) had substantially higher MIC values for all antibiotics (>64 µg/ml) compared to the others. All strains showing resistance against the antimicrobial agents studied were collected in the 1999–2000 period. No resistance was found in either an earlier 1990 or a later 2010 collection years. Telithromycin was not tested for the latest collection in 2000.

Table 17. MIC values and mutations in the macrolide binding site and altered amino acid sequences in ribosomal proteins L4 and L22 among six *H. influenzae* isolates with decreased susceptibility to clarithromycin (Clr), azithromycin (Azm) and/or telithromycin (Tlt).

Isolate	MIC values (µg/ml) ^{a)}			Mutations		
	Clr	Azm	Tlt	23SrRNA ^{b)}	L4 ^{c)}	L22 ^{c)}
Hi66	16	8	8			
Hi198	16	4	>32		T64K	
Hi212	32	8	>32		T64K	
Hi218	16	4	16		T64K	
Hi286	>256	>256	>256	A2058G		
Hi593	64	64	32			E78D, DEL79GP

a) Interpretation criteria according to EUCAST breakpoints (µg/ml): clarithromycin >32 = resistant; azithromycin >4 = resistant; telithromycin >8 = resistant.

b) *E. coli* numbering

c) *H. influenzae* numbering

None of the six resistant strains carried *mef(A)*, *erm(A)*, *erm(TR)*, *erm(B)*, *erm(C)* or *erm(F)* genes.

No mutations in the domain V of 23S rRNA or ribosomal proteins and L4 or L22 were detected in one isolate resistant only to azithromycin (Hi66, Table 14). Three isolates (Hi198, Hi212 and Hi218) showed modifications in the ribosomal protein L4 amino acid sequence. Threonine at position 64 was converted to lysine (T64K) in the L4 protein in all three strains and MIC values were elevated only for telithromycin. In all of these isolates, only one mutation known to confer macrolide resistance was detected in each. In one isolate (Hi286) adenine at position 2058 of 23S rRNA macrolide binding site was replaced with guanine (A2058G, *E. coli* numbering). In this isolate all MIC values exceeded 256 µg/ml. All six alleles of this particular *H. influenzae* isolate were mutated (Haanperä et al., 2005). This mutation was the only resistance mechanism associated with macrolide resistance that was found in this isolate.

Only one strain (Hi593) carried a mutation in the gene encoding for the ribosomal protein L22. The six base-pair (bp) deletion from position 235 forward, caused a replacement of glutamic acid by aspartic acid at position 78 (amino acid position) and a loss of glycine and proline residues at positions 79 and 80 (E78D, DEL79GP, Fig. 19). The MIC values of this isolate were high for all antibiotics. No previously described mechanisms associated with macrolide resistance have been found for this isolate.

H. influenzae Rd KW20

226	GTT	GAT	GAA	GGT	CCT	AGC	ATG	AAA	CGT	GTT	ATG	CCA	CGT	GCT	AAA	270
76	V	D	E	G	P	S	M	K	R	V	M	P	R	A	K	90

H. influenzae hi593

226	GTT	GAT	GA-	---	-- T	AGC	ATG	AAA	CGT	GTT	ATG	CCA	CGT	GCT	AAA	270
76	V	D	D	-	-	S	M	K	R	V	M	P	R	A	K	90

Fig. 19. Nucleotide and corresponding amino acid sequences of L22 riboproteins of *H. influenzae* Rd KW20 (wild-type) and Hi593. Bold letters indicate the nucleotide and amino acid changes in Hi593.

5.2 Association between sulfamethoxazole-trimethoprim use and resistance in *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* (II)

We compared the regional use of the antibiotic to resistance in 21 central hospital districts in Finland to evaluate the effect of sulfamethoxazole-trimethoprim use on resistance in *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*.

Annual, regional consumption figures for sulfamethoxazole-trimethoprim over the 1997–2003 period are shown in Fig. 20 and total consumption in Fig. 21. Consumption had declined in all health care districts until 2002, although not steadily. From 2002 up to the present the use of sulfamethoxazole-trimethoprim increased slightly for most regions, but the total use remained stable. Regional variation in the use of sulfamethoxazole-trimethoprim was considerable (differences between highest annual use and lowest annual use varied from 0.43 to 0.70 DDD/1000 inhabitants/day).

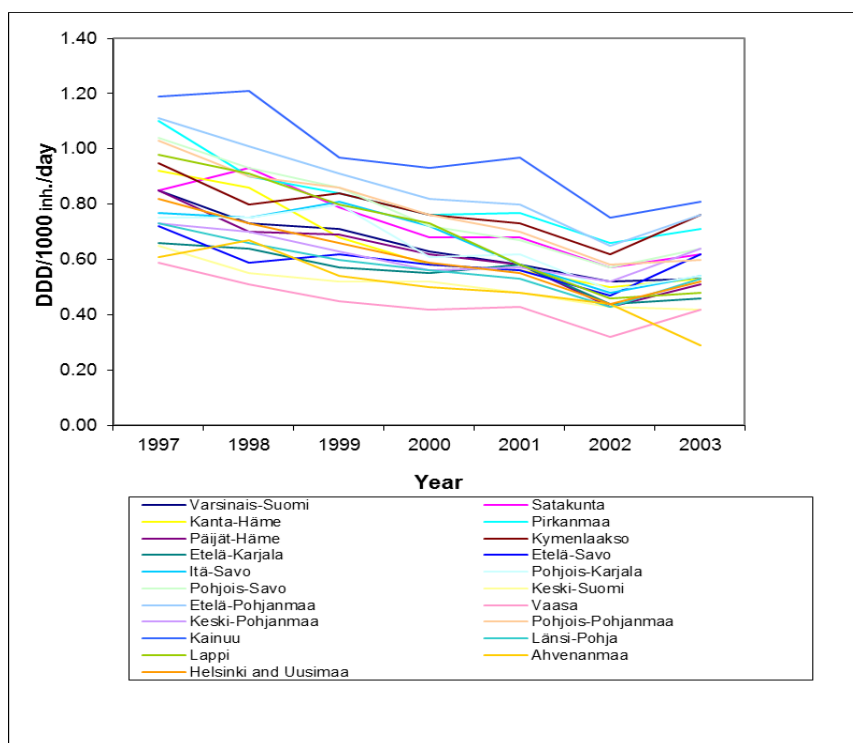


Fig. 20. Regional sulfamethoxazole-trimethoprim consumption in 21 hospital districts in Finland on the 1997–2003 period.

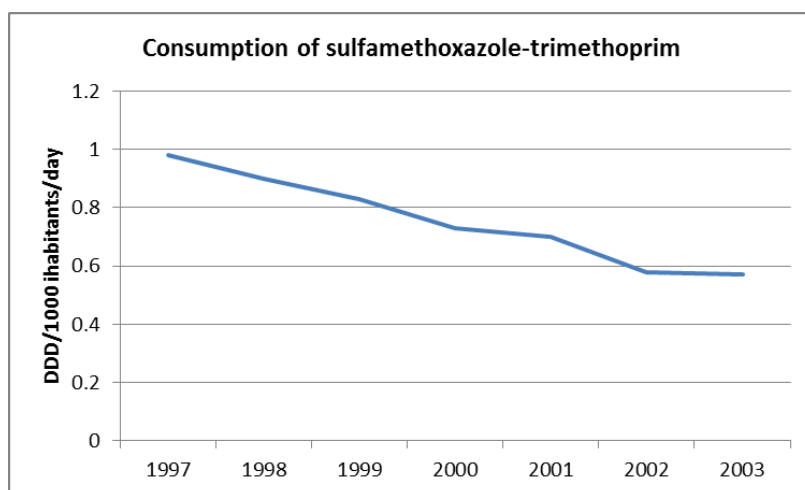


Fig. 21. Total consumption of sulfamethoxazole-trimethoprim in Finland on the 1997–2003 period.

Resistance of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* to sulfamethoxazole–trimethoprim increased sharply from 1988 to 1999 (from 4.5% to 21.4% for *S. pneumoniae*, from 2.7% to 18.1% *H. influenzae* and from 0.2% to 11.4%, for *M. catarrhalis*) in Finland. Resistance started to decline for all three antibiotics after 1990 but started to rise again in 2003 except for *M. catarrhalis* (Fig. 22). In recent years, resistance in *H. influenzae* has increased up to 2014, but slightly declined in 2015. Among *S. pneumoniae* resistance is decreasing and resistance in *M. catarrhalis* –strains is still very low (FINRES, 2015).

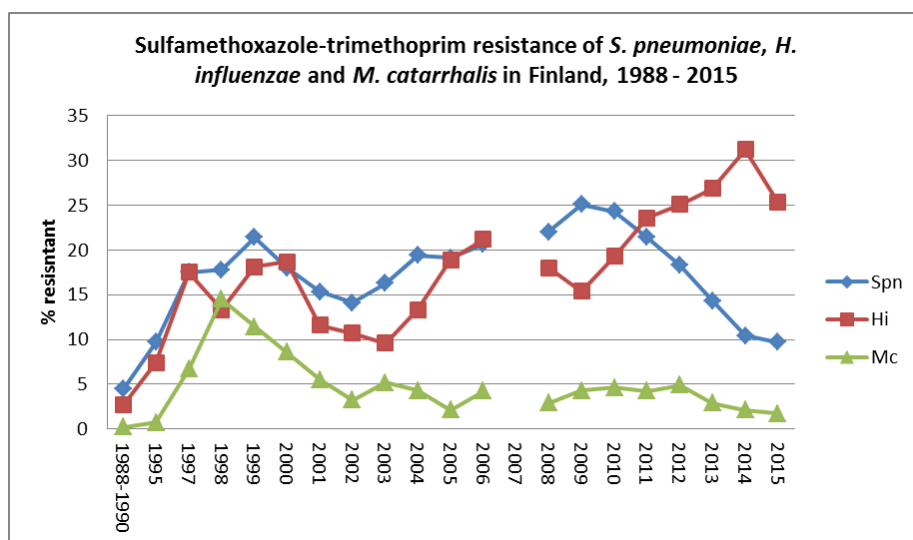


Fig. 22. Sulfamethoxazole–trimethoprim resistance (% resistant) in three respiratory pathogens over the 1988–2014 period. (Spn = *S. pneumoniae*, Hi = *H. influenzae*, Mc = *M. catarrhalis*). From 2007 resistance rates were not available (Nissinen et al., 1995, FINRES 1995–2015).

For evaluating the association of consumption and resistance of sulfamethoxazole–trimethoprim in the three pathogens, we collected regional resistance data from the period 1998–2004. Altogether 23 530 (annual variation 1928–4238) *S. pneumoniae* isolates, 28 320 (3043–5116) *H. influenzae* isolates and 14 138 (1818–2731) *M. catarrhalis* isolates were tested for sulfamethoxazole–trimethoprim during this period. No resistance data were produced from two (2/21) districts (Ahvenanmaa and Kainuu). The remaining 19 health care districts represent 98% of the whole Finnish population (<http://www.kunnat.net/fi/>).

The statistical analyses indicated following associations between sulfamethoxazole–trimethoprim use and resistance: the regional consumption of sulfamethoxazole–trimethoprim seems to have an effect on

regional resistance among *S. pneumoniae* ($p=0.007$). The association was positive, which indicated that the greater the consumption of sulfamethoxazole–trimethoprim, the higher was the resistance of *S. pneumoniae* in the following year. The change of resistance over time was not significant ($p=0.452$) however. The significance in change of resistance over time was borderline for *H. influenzae* ($p=0.051$) but consumption of sulfamethoxazole–trimethoprim failed to explain changes in the level of resistance ($p=0.808$). No significant change in sulfamethoxazole–trimethoprim resistance was seen among *M. catarrhalis* ($p=0.349$) isolates, and again consumption failed to explain the level of resistance ($p=0.744$).

5.3 Quality of resistance data in Finnish microbiology laboratories (III)

A total of 21 out of 25 laboratories reported the internal quality control procedures from the three-year period of 2004 to 2006 inclusive. Of these 15 (71%) included *S. pneumoniae* ATCC49619 in the internal quality control programme in 2004, 17 (81%) in 2005 and 16 (76%) in 2006. The corresponding figures for *H. influenzae* ATCC49247 were 14 (67%) in 2004, 15 (71%) in 2005 and 14 (67%) laboratories in 2006, respectively. The procedures of individual laboratories are presented in Tables 18 A and B.

The qc frequency of these two organisms varied by laboratory. *S. pneumoniae* ATCC49619 was tested annually 0–81 times and *H. influenzae* ATCC49247 0–68 times in the laboratories that performed susceptibility testing for these species (Table 19).

Table 18 A. Antimicrobial agents tested against *S. pneumoniae* ATCC49619 in 21 Finnish laboratories using the disk diffusion and the E-test methods.

Quality control limits approved (FiRe)	Interpretive criteria established for clinical isolates (FiRe)	Antimicrobial agent	Number of results (laboratories)
<i>DD method (concentration of disk, µg)</i>			
No	No	Ampicillin (10)	76 (1)
No	No	Azithromycin (15)	64 (2)
No	No	Cefaclor (30)	1 (1)
No	No	Cephalotin (30)	329 (3)
Yes	Yes	Chloramphenicol (30)	142 (4)
No	No	Ceftriaxone (30)	150 (2)
No	No	Cefuroxime (30)	2 (2)
Yes	Yes	Clindamycin (2)	1607 (15)
Yes	Yes	Erythromycin (15)	1773 (17)
No	No	Levofloxacin (5)	329 (4)
Yes	Yes	Oxacillin (1)	1412 (17)
No	No	Meropenem (10)	98 (1)
Yes	Yes	Moxifloxacin (5)	55 (2)
No	No	Penicillin (10 IU)	837 (8)
No	No	Rifampicin (5)	111 (2)
No	No	Telithromycin (15)	113 (3)
Yes	Yes	Tetracycline (30)	1248 (13)
Yes	Yes	Sulfamethoxazole - trimethoprim (1.25)	1582 (16)
Yes	Yes	Vancomycin (30)	809 (10)
<i>MIC (E-test)</i>			
Yes	Yes	Ceftriaxone	99 (2)
Yes	Yes	Cefuroxime	99 (1)
Yes	Yes	Meropenem	98 (1)
Yes	Yes	Penicillin	138 (4)

Table 18 B. Antimicrobial agents tested against *H. influenzae* ATCC49247 in 21 Finnish laboratories using the disk diffusion method only.

Quality control limits approved (FiRe)	Interpretive criteria approved for clinical isolates (FiRe)	Antimicrobial agent	Number of results (laboratories)
<i>DD method (concentration of disk, µg)</i>			
No	No	Ampicillin (10)	382 (6)
No	Yes	Ampicillin (2)	644 (12)
No	No	Amoxicillin-clavulanic acid (20)	266 (5)
No	Yes	Amoxicillin-clavulanic acid (2)	484 (9)
Yes	Yes	Azithromycin (15)	1036 (13)
No	Yes	Aztreonam (30)	97 (1)
No	No	Cefaclor (30)	358 (6)
No	No	Ceftriaxone (30)	201 (2)
No	No	Cefuroxime (30)	318 (8)
No	No	Doxycycline (30)	99 (2)
No	No	Erythromycin (15)	89 (2)
No	No	Imipenem (10)	97 (1)
Yes	Yes	Tetracycline (30)	1159 (13)
Yes	Yes	Sulfamethoxazole - trimethoprim (1.25)	1273 (16)

Table 19. Annual frequency of quality control for *S. pneumoniae* ATCC49619 and *H. influenzae* ATCC49247 evaluation in 21 FiRe laboratories from 2004 to 2006

Laboratory	<i>S. pneumoniae</i> ATCC49619			<i>H. influenzae</i> ATCC49247		
	2004	2005	2006	2004	2005	2006
1	0	7	34	0	0	0
2	44	48	55	32	35	27
3	0	0	0	52	52	51
4	9	23	29	4	7	10
5	26	26	26	0	0	0
6	0	2	21	0	2	40
7	47	39	37	29	34	35
8	40	37	36	30	34	35
9	0	0	0	0	0	0
10	91	70	82	68	62	57
11	44	44	44	36	45	43
12	13	14	23	13	14	24
13	36	37	0	31	33	0
14	0	0	0	0	0	0
15	0	0	0	0	0	0
16	88	33	33	15	15	18
17	36	35	26	23	20	22
18	52	50	47	0	0	0
19	50	50	17	48	50	32
20	18	24	28	16	24	24
21	19	20	15	12	11	11

A total of 19 different drugs/drug concentrations were included in the DD method quality control programme of *S. pneumoniae* ATCC49619. These included 11 antimicrobial agents or concentrations which were not included in the national guideline (ampicillin azithromycin, cefaclor, cephalotin, ceftriaxone, cefuroxime, levofloxacin, penicillin, rifampicin, telithromycin and meropenem). Of these 11 antimicrobial agents, eight (ampicillin, cefaclor, cephalotin, ceftriaxone, cefuroxime, penicillin, telithromycin and meropenem) are agents for which even the CLSI standard had not provided breakpoints at that time. Sixteen different drugs/drug concentrations were included in the DD quality control programmes of *H. influenzae* ATCC49247. This study also revealed that six drugs (cefaclor, ceftriaxone, cefuroxime, doxycycline, erythromycin and imipenem), which are not recommended in the national standard, were correspondingly tested for *H. influenzae*. Of these, two (doxycycline and erythromycin) were not included even in the CLSI breakpoint tables for *H. influenzae* during 2004 – 2006. In addition, six laboratories performed the ampicillin-test using 10 µg disks instead of 2 µg disks, and they also conducted the amoxicillin-clavulanic acid test using the 20 µg disks instead of 3 µg disks. These procedures were contrary to the recommendations laid down by the FiRe Group.

The susceptibility testing results of *S. pneumoniae* ATCC49619, which used the MH medium (supplemented with 5% defibrinated horse blood) showed good reproducibility. Between 86 and 99% (depending on the

antimicrobial agent) of test results fell within the defined qc-limits provided for the DD method. Laboratories that used IsoSensitest (IS) agar with horse blood performed worse in this analysis (only between 45 to 82% of results were within qc-limits). The numbers for MIC-values were fairly small, and no clear differences between the two media can be seen. The reproducibility of *H. influenzae* testing using HTM agar was very high with tetracycline (95% within limits) and sulfamethoxazole-trimethoprim (90% within limits), but with azithromycin nearly 30% of results fell outside the qc-limits. The lack of confirmed qc-limits for *H. influenzae* ATCC49247 with low-potency ampicillin and amoxicillin-clavulanic acid disks indicated that their performance could not be compared to the established criteria. However, calculating the 2.5 and 97.5 percentiles of their inhibition zones provided a range that would cover 95% of observations (BSAC, 2013), thus the qc-limits for ampicillin 2 µg would be imputed to fall into the range of 6–15 mm and the corresponding qc-limits for amoxicillin-clavulanic 3 µg the limits to be 8–17 mm.

Besides the reproducibility, the categorical validity (S/I/R) of the qc-results was calculated. Reduced susceptibility of *S. pneumoniae* ATCC49619 (NS=not susceptible) was detected as expected with the oxacillin-screening test and also with the E-test method. Moreover, the interpretations for the other antimicrobial agents were nearly correct and no clear differences could be seen between the two media used (Table 20). The reduced susceptibility to ampicillin (due to altered PBP) in *H. influenzae* ATCC49247 was better detected by using the low concentration ampicillin and/or amoxicillin-clavulanate disks. For other antimicrobials the interpretations were nearly as expected (Table 21).

Table 20. Categorical accuracy of the internal quality control results of *S. pneumoniae* ATCC49619 using the DD or E-test method (MIC). Results of two different media are presented separately. R=resistant, I = intermediate, NS = non-susceptible, S=susceptible.

Method ¹⁾	n	R (%)	I (%)	NS (%)	S (%)
Oxa disk MH	950			99.7	0.3
Oxa disk IS	439			99.5	0.5
Pen MIC MH	62	0	100		0
Pen MIC IS	76	0	98.7		1.3
Tcy disk MH	938	0	0		100
Tcy disk IS	290	0	0.3		99.7
Cli disk MH	1051	0	0		100
Cli disk IS	533	0	0		100
SxT disk MH	1137	0.4	1		98.6
SxT disk IS	424	1.7	3.1		95.3
Ery disk MH	1142	0	0		100
Ery disk IS	608	0	0		100
Van disk MH	384	0	0		100
Van disk IS	405	0	0		100

- 1) Oxa = oxacillin, Pen. = penicillin, Tcy = tetracycline, Cli = clindamycin, SxT = sulfamethoxazole-trimethoprim, Ery = erythromycin, Van = vancomycin; MH refers to Mueller-Hinton medium, IS refers to Isosensitest-medium. Breakpoints as defined in the CLSI-standard and/or the FiRe-guideline. The expected results are highlighted in bold lettering.

Table 21. Accuracy of the internal quality control results of *H. influenzae* ATCC49247 in laboratories using HTM-medium and DD method. R=resistant, I = intermediate, NS = non-susceptible, S=susceptible.

Method ¹⁾	n	R (%)	I (%)	NS (%)	S (%)
Amp 2 disk	596	93.1	5.4		1.5
Amp 10 disk	372	20.2	57		22.8
Amc 3 disk	484	79.5	15.9		4.5
Amc 30 disk	218	21.1	0		78.9
Azm disk	1023	0	0		100
Tcy disk	1099	95.9	3.3		0.8
SxT disk	1255	0.1	0		99.9

- 1) Amp = ampicillin, Amc = amoxicillin-clavulanic acid, Azm = azithromycin, Tcy = tetracycline, SxT = sulfamethoxazole-trimethoprim. Breakpoints as defined in the CLSI-standard and/ or the FiRe-guidelines. The expected results are highlighted by bold lettering.

5.4 Susceptibility testing of *Streptococcus pneumoniae* with the E-test and the Vitek2- AST-GP74 test (IV)

Time to results (TTR) of susceptibility testing with the VITEK2® system ranged from 8.5 to 17.5 hours (mean 10 hours , average 10.8 hours) Only one (1/230, 0.4%) isolate was discarded from the study due to insufficient growth.

The CA and FS rates for the VITEK system and E-test are shown in Table 21. Breakpoints for non-meningitis *S. pneumoniae* EUCAST (EUCAST 2016) were used for categorization. Both methods correlated well with the reference method with 98.2% (Vitek2® AST-GP74) and 97.3% (E-test) agreement in S/I/R categories. The lowest CA value was obtained for sulfamethoxazole-trimethoprim by the E-test (88.2%). Numbers of FS results were very low (1% and 1.3%) with both methods.

Total error rates with non-meningitis breakpoints (mE, ME, VME) for the Vitek AST-GP74 were low (1.3, 0.1 and 1.6%). The E-test provided correspondingly 2.2, 0.1 and 4.9% errors. With meningitis breakpoints, the error rates were 0.2, 0.0 and 2.6% for the Vitek2 and 0.7, 0.0 and 0.0% for the E-test.

Total EA for the Vitek2 AST-GP74 was 99.1% and 89.1 % for the E-test.

Table 21. Performance of Vitek2 AST-GP74 and the E-test compared to the broth dilution results for 229 *S. pneumoniae*-isolates. CA = categorical agreement, FS = false susceptible.

	Vitek AST-GP74						E-test					
	CA			FS			CA			FS		
	fraction	n	%	fraction	n	%	fraction	n	%	fraction	n	%
Penicillin	219	229	95.6	2	229	0.9	221	229	96.5	0	229	0.0
Cefotaxime	221	229	96.5	3	229	1.3	219	229	95.6	9	229	3.9
Ceftriaxone	218	229	95.2	9	229	3.9	220	229	96.1	1	229	0.4
Meropenem	228	229	99.6	0	229	0	229	229	100.0	0	229	0.0
Moxifloxacin	228	229	99.6	1	229	0.4	228	229	99.6	1	229	0.4
Levofloxacin	229	229	100.0	0	229	0	ND	ND	ND	ND	ND	ND
Erythromycin	228	229	99.6	1	229	0.4	229	229	100.0	0	229	0.0
Tetracycline	228	229	99.6	1	229	0.4	226	229	98.7	0	229	0.0
Sulfamethoxazole-trimethoprim	220	229	96.1	7	229	3	202	229	88.2	13	229	5.7
Vancomycin	229	229	100.0	0	229	0	229	229	100.0	0	229	0.0
Linezolid	229	229	100.0	0	229	0	ND	ND	ND	ND	ND	ND
Total	2477	2519	98.3	24	2519	1.0	1782	1832	97.2	24	1832	1.3

6 DISCUSSION

6.1 General discussion

This thesis describes the development, evaluation and quality of susceptibility testing methods with particular emphasis and reference to the important respiratory tract pathogens *S. pneumoniae* and *H. influenzae*. Methodological harmonization has proceeded well in Finland due to the activities of the FiRe network. Thanks to this network, a continually increasing comprehensive and uniform body of resistance data of bacterial pathogens exists and it now covers more than two decades. These data were and are used to model the association between antimicrobial use and resistance in these respiratory pathogens and *M. catarrhalis*. A point prevalence study in 2001 indicated that the respiratory tract infections are the most common infections treated in health care centers (74%). Antimicrobial treatment was prescribed for 88% of patients with otitis media, 83% with sinusitis and 71% with acute bronchitis (Rautakorpi et al., 2001). These figures highlight the importance of antimicrobial research on respiratory bacteria.

The degree of antimicrobial resistance among human pathogens has hitherto been low or moderate in Finland. Currently, rising trends are apparent, however, especially in the case of pneumococci (ECDC, 2015 a). One aim of the FiRe network is to maintain resistance of pathogens to antibiotics at low level by guiding the use of antibiotics with the help of limited, but still appropriate and valid susceptibility results. This is done in cooperation with infection diseases specialists.

From the beginning of 2011, Finnish laboratories, like many other European laboratories, have implemented the EUCAST method (EUCAST, 2016) for susceptibility testing of bacteria and this has superseded the national FiRe standard. This switch in guidelines entailed some methodological and interpretative changes. The basic susceptibility testing procedure is still the DD method in most laboratories, although the number of laboratories that use automated methods is increasing. EUCAST has proceeded well in harmonising methods and breakpoints for antimicrobial susceptibility testing of bacteria since 2010, when the first version for disk diffusion method was launched. EUCAST breakpoints are also available for automated susceptibility testing devices with some limitations depending on the system. European countries have widely adopted the EUCAST method and its breakpoints as nearly 90% of laboratories that participate in UKNEQAS distributions in 2014, use EUCAST (ECDC, 2015 a). The results of this study are also highly applicable for current testing standards and practices.

6.2 Evaluation of the data

We evaluated the susceptibility testing method of *H. influenzae* resistance for the FiRe standard (I), whereby 26 laboratories performed testing and reported results. This covers nearly 100% of Finnish clinical microbiology laboratories. Hence, 128–129 repeated tests/disks were received for this study. We used five strains that were obtained from type-strain collections (ATCC) and the UKNEQAS quality control distributions to carry out this evaluation. Unfortunately, the information concerning the altered penicillin binding proteins associated with the BLNAR type of resistance and the genetic confirmation for tetracycline (isolates 1, 3 and 5) or sulfamethoxazole-trimethoprim resistance (isolate 1) were only available for one isolate from ATCC or UKNEQAS databases.

Resistance data used in study II were collected from the annual, nationwide surveillance, FINRES. The material covered data for seven years. Each laboratory provided information on the total number of strains it examined and the numbers of susceptible, intermediate and resistant strains it found. The resistance data are based on the routine susceptibility testing over the whole year for each laboratory and includes all consecutive isolates regardless the site of infection. Multiple isolates of a patient could not be excluded from these datasets. Usually there is only one or two large microbiological laboratories in each hospital district. It has been estimated that more than 90% of bacteriological specimens in a health care district are examined in these “central” laboratories. This is especially true of the laboratories in the public health care sector. Additionally, we had four private laboratories that provided services mainly for private clinics and occupational health care at the time of the study. These four laboratories also provided resistance data to the FINRES surveillance. Geographically the data from private laboratories may be more heterogeneous than the data from public health care laboratories. The location of the laboratory was used as a geographical indicator in this study. Some smaller private laboratories all over Finland do not provide resistance data for the FINRES report. The impact of not having the information of the small laboratories on these FINRES data would be negligible, however. An exact figure of the completeness of the resistance data is not possible, but the population in the health care districts, where the resistance data were available represent about 98% of the total population in Finland. We included the resistance rates based on at least 30 tested isolates per year for statistical reasons; one may assume that the resistance rates among small numbers of tested isolates may be biased. Hence, one can state that the resistance data used is quite uniform and has a good coverage.

Antimicrobial consumption information is collected in Finland as DDD/1000/inhabitants/day (Fimea). In the present study (II) the sum of the quantities of sulfamethoxazole-trimethoprim sold from the two biggest wholesalers to pharmacies was used. Consumption figures from all 21 central

hospital districts were available and hence these consumption data covered the whole country. Hospitals do purchase medicines from other wholesalers and directly from pharmaceutical companies and they may even import drugs directly from abroad. These quantities are estimated to represent, however, only 1% of the total sales of all medicines. In the case of antimicrobial agents this figure has been estimated to be even smaller. Accordingly, the consumption figures used in this study cover at least 99% of the actual sales. There are some conflicting factors that cannot be excluded, such as the quantities derived from unused prescriptions. Additionally, we have no guarantees, that a prescription purchased in one health care district is consumed in the same area. It was mentioned in the Review of literature of this thesis and also pointed out in a review by Fortin et al. (2014), that no single measure gives a complete picture of antimicrobial use. For instance, prescriptions for children are based on weight; this fact is not taken into account when the DDD measure is used.

The quality of the resistance data was evaluated by means of test results for two qc-isolates, *H. influenzae* ATCC49247 and *S. pneumoniae* ATCC49619 (III). Consecutive results were collected from the FiRe laboratories. These two isolates were the concurrently recommended qc-strains for the FiRe standard. Both strains possess “difficult to detect” resistance traits (Tenover et al., 2001, Snell, 1994; Manninen et al., 1998 a, b) and thus offer a good perspective to survey the competence of microbiological laboratories at the national level.

The *S. pneumoniae* isolates used in study IV represent consecutive clinical blood culture isolates (non-selective) and a set of miscellaneous pneumococcal isolates, chosen for decreased susceptibility for penicillin in the initial AST. The number of isolates was 229 and they were collected from one laboratory. According to the FDA guidelines (FDA, 2009) an ideal distribution for methodological evaluations would be 100 isolates of which 50% are susceptible and 50% resistant-strains. However, this kind of distribution may be difficult to collect in practice, especially when resistance is not common. The distributions of S/R isolates expressed as percentages varied in our dataset from 100/0–66.8/33.2% (Table 12). It would be practically impossible to collect 50% resistant strains to all antimicrobial agents tested from the whole country. Repeat isolates from same patient were excluded in case they were obtained less than two months apart.

H. influenzae isolates for searching macrolide resistance mechanisms (unpublished) were obtained from periods in three decades and represent isolates before and after azithromycin and ketolide were introduced. The earliest collection (1988–1991) was selective with regard to specimen site and the age of the patient. The collection from the 1999–2001 period includes isolates from non-selective specimens and all age groups. The latest subset (2006–2011) represents partly selective isolates from children with suspected otitis. Differences in age, infection site and geographical distribution may thus create bias in the results.

6.3 Antimicrobial susceptibility testing methods, changes in international standards and quality of results (I, III, IV)

6.3.1 *Haemophilus influenzae*

6.3.1.1 BLNAR

The detection of non- β -lactamase mediated ampicillin resistance is perhaps the most difficult components of the susceptibility testing of *H. influenzae*. This resistance mechanism has already surpassed β -lactamase mediated resistance in the eastern hemisphere (Ubukata et al., 2003, Witherden et al., 2011, and Park et al., 2013). These BLNAR isolates are also ascending in Europe and North America (Skaare et al., 2014; Shuel et al., 2011). In Finland BLNAR isolates have been found occasionally so far. Consumption of cephalosporins in the 1990s was relatively high in Finland compared to other Nordic countries (Bergan, 2001). The first-generation cephalosporins in particular were used clearly more in Finland than in other European countries in 2009 regardless of the measure used (DID or PID) (Versporten et al., 2011). The high use of cephalosporins in Japan has been associated with elevated BLNAR prevalence for *H. influenzae* (Hasegawa et al., 2006). For these reasons it is important to detect and follow this resistance in Finnish *H. influenzae* isolates.

I have shown in this thesis, that the use of low-concentration disks of ampicillin and amoxicillin-clavulanic acid in susceptibility testing compared to the high-concentration disks that had previously been used, improves the DD method. This was shown with challenge strains in an inter-laboratory study (I) and also in the quality control results of Finnish microbiology laboratories (III). These results are comparable with the findings reported by Zerva and co-workers (1996) and by Sondergaard et al. (2012). As a consequence, the low-concentration disks with HTM medium were adopted for the DD method in the national FiRe standard in 2002. The breakpoints were set at ≤ 13 mm (R) and ≥ 17 mm (S) for both disks. The suggested quality control strain was *H. influenzae* ATCC49247 (BLNAR).

Most laboratories in Finland switched from the FiRe standard to EUCAST from the beginning of 2011 and changes were made for AST of *H. influenzae*. The EUCAST method utilises MH-F medium instead of the formerly used HTM. The use of a low concentration ampicillin disk (2 μ g) but high concentration amoxicillin-clavulanic acid disk (30 μ g) was the method of choice in the first EUCAST guideline versions (1–1.3). Version 2.0 (published

1.1.2012) stipulated a low concentration amoxicillin-clavulanic disk (2+1 µg) to replace the high concentration disk previously used and a novel screening method to detect ampicillin resistance (penicillin disk, 1 unit) was introduced. These changes were shown to have best sensitivity and accuracy in identifying *H. influenzae* strains with PBP3 mutations. This method was not suitable for ampicillin resistant, β-lactamase positive (BLPAR) isolates, however, and therefore a cefuroxime (5 µg) disk was suggested instead (Skaare et al., 2015). That study also included a warning about ignoring a hazy growth within inhibition zones.

The CLSI guideline still relies upon high-concentration ampicillin and amoxicillin-clavulanic acid disks and HTM medium in AST of *H. influenzae* (CLSI, 2015). The benefit of using the HTM medium is a more distinct endpoint compared to more nutrient-rich media such as MH-F (Barry et al., 2001).

The tetracycline results in our study indicate that a part of the susceptible population was misclassified as belonging to the intermediate category. The sulfamethoxazole-trimethoprim tests, however, showed good accuracy. The histogram is clearly bi-modal and the existing breakpoints do not split the susceptible and resistant populations (Unpublished, Fig. 17 and 18).

Switching from the FiRe standard to EUCAST in 2011 has not changed the observed resistance rates of ampicillin, amoxicillin-clavulanate and tetracycline in *H. influenzae* in Finland. The observed sulfamethoxazole-trimethoprim resistance instead is higher for all age groups in the EUCAST era. Resistance was from 18.1% in 2008 to 21.9% in 2010 (during the pre-EUCAST era) and has varied between 23.6 and 31.2% in the EUCAST era (FINRES, 2015). However, there is no direct evidence to suggest that the observed rise in resistance is a consequence of methodological changes or different breakpoints following the implementation of EUCAST.

6.3.1.2 Macrolides

We examined 713 *H. influenzae*-isolates from periods belonging to three different decades that had been submitted by various laboratories in Finland. Only 6 out of 713 (0.8%) strains were resistant for one or more macrolides according to the CLSI breakpoints or current EUCAST breakpoints. The breakpoints used today have been set basically to differentiate wild-type strains, and not for the classification of *H. influenzae* into susceptible, intermediate or resistant types. The correlation between macrolide MIC values of *H. influenzae* and clinical outcome is poor due to intrinsic efflux possessed by most *H. influenzae* strains (Peric et al., 2003; Bogdanovich et al., 2006). Despite the poor correlation it would be of interest to examine, whether the well-known resistance mechanisms in other bacteria that share the same habitat have spread to *H. influenzae*.

We found no mobile macrolide resistance genes in our isolates. This finding agrees with the results obtained by Peric et al. (2003) from the

Alexander-project (Felmingham et al., 2005; Atkinson et al., 2015) from Australia. On the other hand, Roberts and colleagues in 2011 and in 2015 found the mobile *erm* and *mef* genes in *H. influenzae* isolates in North-America among children with CF (Roberts et al., 2011; Roberts et al., 2015). 50% of patients were exposed to azithromycin during the study and presumably all children had a previous history of macrolide therapy. Our material is non-selective with regard to patients' previous exposures to macrolides, which is a similar condition that prevailed in the study by Peric and co-workers. In the Australian study, however, 15.7% of patients were known to have had a significant macrolide exposure prior to culture of *H. influenzae*. In total resistance rates were low (< 5 %) in that study except of those isolates with preceeding exposure, where it was 25.5%. It is likely, that among selective populations (with preceding exposure), horizontal transfer of macrolide resistance in *H. influenzae* may occur. However, among populations without such burden this is not a probable perspective. This conclusion endorses avoiding heavy use of macrolides in the treatment of community acquired infections. CF is a rare disease in the Finnish population (Halme et al., 2006); hence patients with long azithromycin therapy are infrequent.

Four different mutations were detected in the Finnish macrolide resistant *H. influenzae* isolates. The transversion of adenine to guanine in position 2058 of 23SrRNA (A2058G) has been reported earlier in other respiratory bacteria and *H. influenzae* (Jalava et al., 2004; Pihlajamäki et al., 2002; Peric et al., 2003). We found this mutation in one isolate (hi239), which showed high resistance to all macrolides, including telithromycin. In pneumococcal isolates telithromycin has remained active, when this mutation is present (Farrell et al., 2003; Van Eldere et al., 2005). Three isolates carried previously described (Peric et al. 2003) mutations in the L4 ribosomal protein, which led to the one amino acid substitution (threonine to lysine at position 64). All of these strains were resistant to telithromycin and one strain was resistant to azithromycin as well. Finally, we found a novel mutation in the ribosomal protein L22, which was associated with high level resistance to all macrolides. The six base pair loss caused one amino acid substitution and loss of two amino acids in the highly conservative region of the L22-protein (E78D, DEL79GP). No other previously described resistance mechanisms were detected and therefore this mutation is likely to be the molecular mechanism behind macrolide resistance in this particular isolate.

6.3.2 *Streptococcus pneumoniae*

A high level of agreement between an automated susceptibility test-system (VITEK2® AST P74-card) and the BD method (Sensititre®) was found in this study. All calculated parameters: CA, EA and number of errors were within limits, which the FDA (2009) has defined for approval of a novel susceptibility testing system with the non-meningitis breakpoints set by

EUCAST. The FSR rate was very low, as well. The FDA specifications are not, however, fully applicable to this study, because the bacterial population did not meet the ideal distribution (50% susceptible, 50% resistant). It is stated in the FDA document, however, that this may be a rare situation when consecutive clinical isolates are used. It is also worth noting that, several reports around the world indicate that resistance to vancomycin and linezolid does not occur among pneumococci.

Automatic susceptibility testing methods are used by many microbiological laboratories, mostly for gram-negative rods and staphylococci. When these fully automated systems have been evaluated, problems have been observed with *S. pneumoniae*. The discrepancies have been mostly classified as minor errors in many evaluations (Ligozzi et al., 2002; Chavez et al., 2002; Goessens et al., 2000; Abele-Horn et al., 2006). A cross-comparative study conducted by Mittman and co-workers (2009), reported that the Phoenix® system produced fewer VMEs (n=1) than the VITEK2®-system (n=7) among 311 isolates tested. CA, EA and ME were, however, very similar between both methods and thus met the minimum requirements of the FDA (2009). The difference in the number of VMEs might be a reflection of a shorter TTR of the VITEK2®-system (average 9.8 hours) compared to that of the Phoenix® -system (average 12.1 hours). The BD method was used as a reference for the MIC values.

The high VME number (2.6%) for penicillin with the meningitis breakpoints is of concern regarding the Vitek2 method. This is in agreement with the work of Charles and co-workers (2016), who compared four commercial methods and showed that with CLSI breakpoints for the E-test, M.I.C.E (MIC Evaluator®) and VITEK2® provided unacceptable error rates, especially with the meningitis-breakpoints. In addition, these methods tended to report lower MIC values for β -lactams than that obtained by the reference BD method.

The aim of this study was to validate an automated method to replace the standardized DD method for *S. pneumoniae*. This would promote the automatization in clinical microbiology laboratories. These results indicate that the VITEK2®-AST-GP74 is a decent option for this purpose. However, the isolates from cerebrospinal fluid samples must be tested by other methods. The utilization of rapid laboratory technology with positive blood cultures reduces the use of broad-spectrum antibiotics and treatment of contaminants especially, when rapid identification of the pathogen is combined with antimicrobial stewardship (Banerjee et al., 2014; Sothoron et al., 2015).

6.3.3 Quality assurance

Assessment of laboratory performance in susceptibility testing is important for two reasons: 1. the results have direct influence on patient care 2. reliable data are needed for surveillance studies of antimicrobial resistance. Good

laboratory practices call for laboratories to participate in external quality assurance schemes (EQA) and monitor all susceptibility testing methods they use by defined internal qc-programs (IQA).

We examined the IQA results of Finnish laboratories to get a picture of the national FINRES data of *S. pneumoniae* and *H. influenzae*. The study was conducted in the 2004–2006 period, i.e. the FiRe standard era. Two findings were unexpected. First, 3 out of 21 laboratories in study period did not include the recommended pneumococcal quality control strain in the IQA programme and 4 out of 21 laboratories did not use the recommended *H. influenzae* strain. However, all these laboratories produced susceptibility results for clinical *S. pneumoniae* and *H. influenzae* isolates. Second, some of the laboratories that participated in this study, tested and reported results for antimicrobial agents without defined breakpoints for clinical isolates or ranges and targets for the specified qc-strain. This practice has been reported earlier in the USA (Doern et al., 1999). Moreover, eight out of 21 laboratories in our study that tested penicillin susceptibility of *S. pneumoniae* used the high-strength 10 µg penicillin disk and a few laboratories tested ceftriaxone, cefuroxime and meropenem susceptibility with the DD method. Additionally, only a few laboratories monitored the results of the MIC methods for *S. pneumoniae* although they examined the isolates obtained from invasive infections. ‘Non-compliant’ disks of ampicillin 10 µg and amoxicillin-clavulanic acid 30 µg were also used for *H. influenzae*. Erythromycin was also tested in two of the 21 laboratories, although its activity against *H. influenzae* is low. Additionally, ceftriaxone and cefuroxime were tested with the DD method. However, one can estimate from the data collected, that >90% of the national surveillance data was produced with methods with high compliance with the national guidelines.

The present study of the IQA results in Finnish laboratories confirms the earlier finding (I), that non-β-lactamase mediated chromosomal ampicillin resistance in *H. influenzae* is detected with better accuracy, when low concentration of ampicillin (2 µg) and/or of amoxicillin-clavulanic acid (3 µg) disks are used in the DDD method. Several wild type quality control strains (ATCC9334 and NCTC 8468) have been recommended in previous versions of the EUCAST standard for *H. influenzae* testing. The current qc-isolate is ATCC49766. The extended qc recommendation includes an isolate with the PBP3 mutation, ATCC49247 (EUCAST 2016). This β-lactam resistant isolate is included in the CLSI as a regular control (CLSI 2015). In view of our results (III) the inclusion of one BLNAR qc-strain along with the wild-type strain is useful to confirm the capacity of laboratories to find the difficult-to-detect, and possibly emerging ampicillin resistance in *H. influenzae*. So far, no qc-data are available on the capacity of penicillin disks in routine laboratory work in Finnish laboratories to detect correctly the isolates with altered PBP3 proteins, although this has been well documented elsewhere (Skaare et al., 2015).

S. pneumoniae ATCC49619 is the recommended qc-strain for both prevailing AST standards (EUCAST and CLSI). There is a paucity of data on the effect of medium to susceptibility results among pneumococci. A study by Jones and colleagues (1996), however, observed that the choice of susceptibility test medium (Iso-Sentitest/Muller-Hinton) affected the expression of macrolide-lincosamin-streptogramin (MLS) resistance in *S. pneumoniae*.

The assessment of laboratory performance should be studied anew with current practices. However, as mentioned earlier, the primary *H. influenzae* qc-strain is different from that used in this study and it has also been altered during the EUCAST era. Therefore, there is probably not enough data of this aspect to make any firm conclusions about the present state.

6.3.4 Association between antimicrobial resistance and consumption (II)

The sulfamethoxazole-trimethoprim resistance fluctuated in Finland among the major respiratory tract pathogens in our study period 1998–2004. A remarkable decline in resistance is seen among pneumococci since 2010. The number of multiresistant isolates has also declined. Resistance has risen among *H. influenzae* in all age groups and been quite persistent among *M. catarrhalis* during the same period (FINRES, 2015).

A statistically significant association between sulfamethoxazole-trimethoprim consumption and resistance in *S. pneumoniae* was shown in this study but not among other respiratory pathogens (II). The trend in sulfamethoxazole-trimethoprim consumption, and that of trimethoprim (Tmp) alone, are declining (www.fimea.fi). When the current resistance figures are examined, one has to bear in mind, that the transition to the EUCAST method in Finnish laboratories may have influenced the resistance rates since 2011 due to the methodological changes and different breakpoints that followed the implementation.

The effect of changes in antimicrobial use on resistance is a complex issue. As discussed in the Review of literature section (2.4.1.), several studies have shown a positive connection between antimicrobial use and resistance. On the other hand, there are studies that show no association or even a negative association. Clonal spread of resistant strains is probably one reason to explain persistent resistance despite a restriction in antimicrobial use (Arason et al., 2002). We do not have genotypic information of the isolates used in this study. Hence, we do not know, whether the spread, persistence or disappearance of resistant clones has affected the results. Statistical data of resistance was the method of choice in this study instead of a subset of bacterial strains. With this approach we were able to collect the data from a fairly long time span and from a wide geographical area. We also lack information of multiresistance among the bacterial strains used in this study. Multiresistance is relatively common especially among pneumococci in

Finland (3.0% for four antimicrobial agents and 7.4% for two antimicrobial agents in 2015) and in Europe as a whole (FINRES 2015, ECDC 2015a). Dual resistance was associated with serogroups 19, 14 and 6 in 12 European countries.

Multiresistance may be one reason to explain the persistence or increase in resistance despite declining consumption of one antimicrobial agent. There is no recent data of multiresistance among *H. influenzae*, so conclusions of its persistent or rising resistance to sulfamethoxazole-trimethoprim cannot be evaluated from this point of view. The lack of clonality among multiresistant *H. influenzae*, in the 1990s was shown in two studies (Fuste et al., 1996; Gazagne et al., 1998).

Sulfonamide resistance in *E. coli* has remained at the same level in the UK despite a 97% reduction in its consumption. Several factors may have contributed to the persistence of resistance, such as the use of sulfonamides in veterinary medicine or the co-selection of sulfonamides-resistance by other antimicrobial agents. An interesting result reported by Enne et al. (2004) is that not all plasmids that conferred sulfonamide resistance reduced the fitness of the host cell. Indeed, they found a plasmid, p9123, which actually improved it.

7 CONCLUSIONS AND FUTURE CONSIDERATIONS

The key findings of the present study are :

(1) The detection of *H. influenzae* bacterial strains that carry mutations in the PBP 3-proteins (BLNAR-type resistance) is more accurate with the DD method when low-strength ampicillin (2 µg) and amoxicillin–clavulanate (3 µg) disks (I, III) are used instead of the corresponding high-concentration disks (10 and 30 µg) (sensitivities 92 and 91% for low-strength compared to 71 and 41% for high-strength). This resistance is of importance, because these drugs are the first line treatment of acute otitis media in children and other upper respiratory tract infections. Globally this type of resistance has increased and is often suggested to be a consequence of the high use of cephalosporins. In Finland, cephalosporins are among the three most prescribed antimicrobial agents in outpatient care. This highlights the necessity to use best practices in the β -lactam susceptibility testing of *H. influenzae*.

(2) Regional changes in sulfamethoxazole–trimethoprin consumption were shown to have a positive association with regional resistance rates among *S. pneumoniae* but not among *H. influenzae* and *M. catarrhalis* (II). Changes in resistance were not significant over time in the study period of seven years. Genetic analyses of representative subsets of these bacteria are needed to find the mechanisms for these observations. Studies on the link between antimicrobial use and resistance of bacteria are also needed to find which agents cause the least resistance pressure among pathogenic bacteria. Many studies show a link between antimicrobial use and resistance for different population levels. An interesting point of view would be to study this association at the individual level.

(3) Analysis of quality control results with susceptibility testing in Finnish microbiology laboratories showed that by using standardised methods, the results are highly reproducible. Two quality control strains, *H. influenzae* and *S. pneumoniae* with challenging, but important resistance traits, were correctly detected with excellent cover. However, noncompliance with the standardised methods was seen in many laboratories. A corresponding analysis of susceptibility testing in the EUCAST era would also be useful.

(4) The automated susceptibility testing method (VITEK2®) may offer an alternative for the testing of *S. pneumoniae* in clinical laboratories. The obvious benefit would be that results would be obtained much faster than the current conventional manual methods (time to results a mean of 10 hrs compared to 18–24 hrs for manual methods). However, the discrepant

results for antimicrobial agents used for the treatment of meningitis, obtained by this method are of note and caution is required in adopting new methods.

(5) Wild type *H. influenzae* with respect to macrolides, azalides and ketolides, have dominated in Finland up to 2011, at least among children. A very small number of resistant strains were found, and none of them carried the genes mobile resistance genes (*mef* and *erm*) found in other bacterial species. However, three types of mutations in the V domain of 23S rRNA and ribosomal proteins L4 and L22 were detected. The lack of mobile genes conferring resistance is likely due to fairly low and steady consumption of these antibiotics in Finland.

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